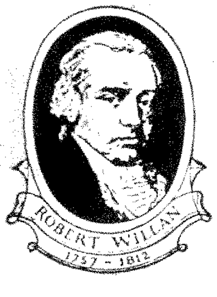

British Journal of Dermatology

CUK – H06078 – 34 – P25448.

Photodamage in the Community [illegible]

Phase of Long-Term Topical [illegible]

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Published for the
British Association of Dermatologists
by Blackwell Scientific Publications

[illegible]

British Journal of Dermatology

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DESPATCH. The Journal is despatched within Europe by surface mail, to other continents by various forms of air-speeded delivery: to the U.S.A.* by air freight for forwarding by second class post, to India by air freight for guaranteed local delivery, and to all other countries by Accelerated Surface Post.

* Second-class postage paid at Rahway, NJ. Post Master, send address changes to *British Journal of Dermatology*, c/o Mercury Airfreight International Inc., 2323 EF Randolph Avenue, Avenel, NJ 07001, U.S.A.

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This journal is printed on acid-free paper.

P,25,448

ABC

Printed in Great Britain at The Alden Press, Oxford

Photodamage in the Community and the Place of Long-Term Topical Retinoid Therapy

Based on communications presented at an official satellite
symposium of the 2nd Congress of the European Academy of
Derm[illegible] and Venereology,
Athens, [illegible], 10–13 October 1991

Sponsored by an educational grant from
Cilag International Commercial Development

Editor
Professor S.S.Bleehen
Department of Dermatology, Royal Hallamshire Hospital, Sheffield, UK

Guest Editor
Professor R.Marks
Department of Dermatology, University of Wales College of Medicine, Heath Park,
Cardiff, UK

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British Journal of Dermatology (1992) 127, Supplement 41, 1.

Does photodamage matter?

The clinical relevance of photodamage has been questioned by many dermatologists in recent years. Solar damage causes skin malfunction and disability, and these should be the legitimate concerns of dermatologists. Photodamaged skin provides less mechanical protection and support than does normal skin. The blood vessels of the dermis, with less physical support, become telangiectatic and more vulnerable to trauma, causing 'senile purpura'. Such lesions should really be called 'solar purpura', and are also seen in the wasted skin of patients treated with corticosteroids. Chronically solar-exposed skin also has fewer Langerhans cells than non-exposed skin, and is less able to mount a delayed hypersensitivity response to foreign antigens with which it comes into contact. While this may seem to be useful in the short term, as far as allergic contact dermatitis is concerned, we should be aware of the potential long-term significance. This defect implies the free permeation of toxic and/or carcinogenic substances and, indeed, an inability to mount an attack on neoantigens of mutated cells, enabling abnormal clones of the latter to survive and form neoplastic lesions. Whether or not this is the major mechanism, a multiplicity of skin cancers develop in photodamaged skin. Indeed, the entire exposed skin of severely photodamaged subjects seems to show dysplastic changes so that solar keratoses should really be regarded as the 'tip of the preneoplastic iceberg'.

Apart from these examples of abnormal function in photodamaged skin, the cosmetic disability is real and the alterations are of major concern to those who have them. Should not the fine lines, the yellow appearance, the pigmented mascules, the stellate scars and the other changes of photodamage be regarded in the same light as the changes of rosacea or acne? Up until recent times, we have had a mistaken belief as to the inevitability of the changes of photodamage. However, we now know better and we can no longer continue to trivialize the clinical significance of photodamage and regard it as a condition beyond treatment.

Our increased longevity, compounded by increased solar exposure resulting from changing social patterns, has produced an increase in both melanoma and non-melanoma skin cancers. Ozone depletion may well aggravate this problem quite soon.

Yes, photodamage does matter, and may well matter more in the near future. This supplement is based on a Cilag-sponsored symposium on 'Photodamage in the Community and the Place of Long-Term Topical Retinoid Therapy' held at the EADV (October, 1991), and is devoted to the clinical manifestations, assessment and treatment of photodamaged skin.

R.Marks
Department of Dermatology
University of Wales College of Medicine
Health Park, Cardiff, UK

British Journal of Dermatology (1992) **127**, Supplement 41, 2–6.

Ozone depletion and its effects on human populations

R.RUSSELL JONES
St John's Dermatology Centre, St Thomas's Hospital, London, UK

Summary

Concern about the ozone-depleting potential of man-made chemicals has led the United Nations to control their usage. Under the present arrangements, fully halogenated chlorofluorocarbons, halons and carbon tetrachloride will be phased out by the year 2000, and methylchloroform by the year 2005. Even so, atmospheric chlorine is expected to rise to seven times its natural level, so further ozone losses and greater ultraviolet B (UVB) exposure can be expected over heavily populated areas of the globe. Calculating changes in skin cancer incidence is a two-stage process which must take account of the increase in biologically effective UVB that results from an ozone loss of 1% (optical amplification factor, OAF) and the percentage increase in skin cancer incidence that results from a 1% increase in annual UV dose (biological amplification factor, BAF). Epidemiological data provide a BAF value of approximately 1·7 for basal cell carcinoma and 3·0 for squamous cell carcinoma. The absorption spectrum of ozone and the increased carcinogenic impact of UV radiation around 300 nm results in a value for OAF of approximately 1·6%; thus a 10% loss of ozone, if sufficiently sustained, would eventually increase the incidence of basal and squamous cell carcinomas by almost 30% and 50%, respectively. Dermatologists, therefore, need to look carefully at the environment in order to safeguard the health of future generations.

Ozone, a gas that comprises three atoms of oxygen, is present throughout the atmosphere, but is found in greatest concentrations in the stratosphere. Total column ozone refers to the total amount of ozone present vertically throughout the atmosphere, and includes the troposphere (<15 km), the stratosphere (15–50 km) and the mezosphere (50–85 km). High-altitude ozone is generated by the action of ultraviolet (UV) radiation on molecules of oxygen

$$O_2 + h\nu \rightarrow O + O$$

where $h\nu$ is a quantum of radiation at a wavelength of less than 243 nm and

$$O + O_2 \rightarrow O_3$$

Tropospheric ozone is generated by the action of UV radiation on molecules of nitrogen dioxide

$$NO_2 + h\nu \rightarrow NO + O$$

and

$$O + O_2 \rightarrow O_3$$

Formation of tropospheric ozone is accelerated by sunlight acting on atmospheric pollutants, particularly hydrocarbon emissions from car exhausts and nitrogen oxides produced by transport and industry.

At high concentrations, tropospheric ozone has deleterious effects on human health. It directly affects respiratory function, and the World Health Organization recommends an 8-h maximum exposure of 60 parts per billion (ppb). By contrast, ozone in the stratosphere and elsewhere plays a critical role in protecting living organisms from the harmful effects of UV radiation. Ozone is broken down by sunlight

$$O_3 + h\nu \rightarrow O + O_2$$

where $h\nu$ is a quantum of energy at a wavelength of less than 315 nm. This reaction virtually eliminates UVC radiation (wavelength <280 nm) from reaching the surface of the planet, and greatly reduces the amount of UVB radiation (wavelength 280–320 nm).

The process by which ozone is broken down can be greatly increased by the catalytic effect of free radicals

$$\cdot X + O_3 \rightarrow XO + O_2$$

and

$$XO + O \rightarrow \cdot X + O_2$$

where $\cdot X$ is a free radical, such as $\cdot Cl$, $\cdot Br$, $\cdot H$, or $\cdot NO$.

Without the protective effect of ozone, terrestrial

Correspondence: Dr R.Russell Jones, FRCP, St John's Dermatology Centre, St Thomas's Hospital, London SE1 7EH, UK.

ecosystems would not have evolved in the way that they have.[1] Simple aquatic organisms, such as zooplankton, are particularly sensitive to charges in ambient UV radiation. Terrestrial ecosystems may also be affected: for example, approximately two-thirds of agricultural plants tested showed decreased crop yields when exposed to increasing levels of UVB radiation.

The effects of stratospheric ozone depletion on humans can be divided into direct and indirect effects.[2,3] Most of the direct effects result from increasing UV radiation reaching the skin or the eye. These effects may be acute (sunburn and acute keratitis), or chronic (photoageing, skin cancer and cataracts). In addition, there may be increased susceptibility to cutaneous infections, such as herpes simplex, and decreased effectiveness of vaccination programmes due to the immunosuppressive effect of UVB radiation. The effect of ozone depletion on the incidence of photodermatoses is more complex. The effects of UVB radiation will be aggravated, whereas UVA-induced dermatoses, such as polymorphic light eruption, may lessen since the acute effects of exposure to UVB radiation will cause subjects to protect their skin from burning before significant UVA exposure has occurred.

Indirect effects of ozone depletion are no less important, but may be more difficult to quantify. As stratospheric ozone declines, more UV radiation reaches the troposphere, thus increasing the formation of ground-level ozone, resulting in an increase in respiratory ailments. Another indirect effect concerns food supplies: tropospheric ozone, as well as UVB radiation, reduces crop yields in sensitive species and is itself a powerful 'greenhouse gas'. The combined effect of increasing UVB radiation, high concentrations of tropospheric ozone and global warming could exert major deleterious effects on agricultural production. There is no reason to think that industrialized nations will be immune to these effects, which could far outweigh the direct effects on human health.

Man-made chemicals

Concern about the ozone-depleting potential of man-made chemicals was first expressed in 1974, when a paper in *Nature* predicted catalytic destruction of stratospheric ozone by free radicals generated by the action of sunlight on long-lived chlorine-bearing compounds.[4] Of chief concern were the chlorofluorocarbons (CFCs), which were widely used at the time in aerosols, food packaging, insulation, refrigeration and air-conditioning systems. Despite these concerns, usage of CFCs continued to increase. New applications were found, such as the cleaning of electronic circuitry, so that by 1985 annual production had reached 1000 kilotonnes. Since CFCs have very long atmospheric residence times (110 years in the case of dichlorodifluoromethane), atmospheric concentrations were increasing by 5–10% per annum.

Table 1. Chemicals included in the revised Montreal Protocol (1990)[5]

Substance	Chemical name	Ozone-depleting potential	1985 world production (ktonnes)
Group 1			
CFC 11	Trichlorofluoromethane	1·0	370
CFC 12	Dichlorodifluoromethane	1·0	470
CFC 13	Trichlorofluoromethane	0·8	160
Group 2			
Halon 1211	Bromochlorodifluoromethane	2·7	10
Halon 1301	Bromotrifluoromethane	11·4	10
Group 3			
CH_3CCl_3	Methylchloroform	0·15	560
CCl_4	Carbon tetrachloride	0·9	80

Bromine-bearing compounds (halons) are also a major concern. Although produced in smaller quantities, their ozone-depleting potential is greater than that of the fully halogenated CFCs. Bromotrifluoromethane, for example, has an ozone-depleting potential of 11·4 (i.e. 11·4 times greater than that of the fully halogenated CFCs, such as trichlorofluoromethane or dichlorodifluoromethane). A large pool of halons is currently banked in fire-fighting systems world-wide awaiting release at some future date.

The important ozone-depleting chemicals, together with their ozone-depleting potential and world production figures for 1985, are listed in Table 1.

International controls

Under the auspices of the United Nations Environment Programme (UNEP), attempts have been made to limit the production and use of ozone-depleting chemicals.[5] The latest proposals are that CFCs, halons and carbon tetrachloride be phased out by the year 2000, and methylchloroform by the year 2005. These proposals have been ratified by the major CFC-producing nations, but there is a 10-year derogation for developing nations, and compliance may be incomplete: for example, India has still to ratify the agreement. Even assuming that compliance is complete, further major changes in atmospheric chemistry can still be expected.

Prior to CFC usage, concentrations of chlorine in the atmosphere were approximately 0·6 ppb. The ozone hole over Antarctica appeared when concentrations reached 1·5–2 ppb, and currently concentrations are above 3 ppb and will peak at 4·2 ppb, or higher, before starting to decline.[6] The consequences of allowing atmospheric chlorine to reach seven times its natural level cannot be predicted with certainty. Huge losses of ozone over Antarctica during springtime were not anticipated by any of the scientists involved in atmospheric ozone research, and the observed rate of loss of ozone over the northern hemisphere (4–5% per decade) is greater than that predicted by atmospheric models.[7] It would be wrong to suppose that ozone depletion will proceed in an orderly fashion.

Stricter controls are needed to prevent further rises in atmospheric chlorine, and it is particularly unfortunate that the phase-out date for methylchloroform has been delayed until 2005. Methylchloroform is produced in larger quantities than any other compound with ozone-depleting potential, but has a short atmospheric residence time (8 years), and an immediate ban would be the most effective way of rapidly reducing atmospheric chlorine concentrations.

Quantification of effects

Quantification of future effects on human health is not an exact science, since it depends upon a large number of variables, including the protective measures that future generations may take. Pollutants in the lower atmosphere and changing climatic conditions may also affect the amount of UVB radiation that reaches the surface of the planet. Calculations are, therefore, made on the assumption that all other factors remain equal. The problem is well illustrated by ground-based observations in North America between 1974 and 1985, which showed no evidence of an increase in UVB radiation at a time when ozone concentrations are reported to have declined.[8] Most of the UV-monitoring devices, however, were located close to urban areas and local pollution episodes may have affected the readings more than did changes in the total column ozone. By contrast, observations recorded at a high mountain location in Switzerland have shown an 11% increase in UVB radiation between 1980 and 1989.[9]

Calculating likely increases in the incidence of skin cancer is a two-stage process, which must take into account the increase in biologically effective UV radiation that results from an ozone loss of 1% (i.e. the optical amplification factor, OAF) and the percentage increase in the incidence of skin cancer that results from each increment in annual UV radiation dose (biological amplification factor, BAF).[10] The value of BAF will vary according to the type of skin cancer under consideration, and is derived from epidemiological data that correlate the incidence of skin cancer with geographical latitude and, hence, UV dose. The OAF value depends upon the absorption spectrum of ozone, the zenith angle of the sun and the system used to determine the relative biological effect of UV radiation at different wavelengths.

Before the action spectrum for photocarcinogenesis was fully elucidated, various surrogates were used. Action spectra based on UV-induced DNA damage, for example, yield higher OAF values than those based on erythema in animal models. The Robertson–Berger

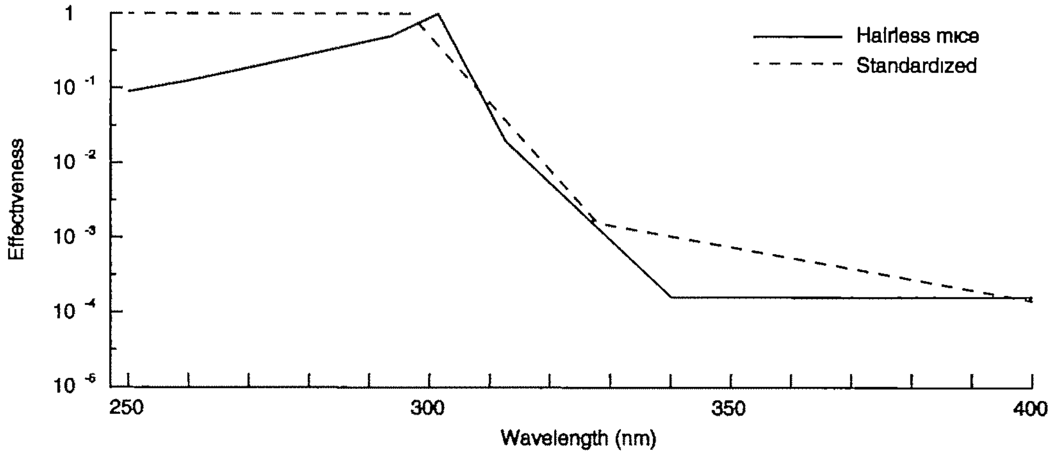

Figure 1. Action spectra for photocarcinogenesis in hairless albino mice and a standardized action spectrum for UV radiation-induced erythema. Reproduced with permission from Van der Leun.[13]

meter represents a third method of weighting UV dose. This employs a magnesium tungstate sensor to monitor radiation in the UVB range, which is then weighted according to an action spectrum based on skin erythema in humans, but attaches greater weight to longer wavelengths.[11] The ability of these different systems to predict the tumorigenic capacity of UV radiation using the hairless mouse model has been investigated by Cole *et al.*[12] The authors concluded that the DNA action spectrum tended to overestimate, whereas the Robertson–Berger meters tended to underestimate the carcinogenic impact of UVB radiation. More complete data have been provided at the University of Utrecht by Van der Luen,[13] who has defined more precisely the action spectra for the production of squamous cell carcinoma in hairless albino mice, and demonstrated that this corresponded quite closely to a standardized action spectrum for UV radiation-induced erythema. Maximum effect in terms of carcinogenesis occurs with radiation of wavelengths around 300 nm, and falls off rapidly at longer wavelengths, decreasing by four orders of magnitude at 340 nm (Fig. 1). The relative biological effect, interestingly, also decreases towards the UVC range, not because UVC radiation is less damaging to DNA, but because of the stronger absorption of shorter wavelength UV radiation by the stratum corneum. An OAF of 1·6 has been derived from these data. At zenith angles of more than 60°, the OAF value is somewhat lower, but still exceeds unity.

The value of BAF will depend upon the skin tumour under consideration and the fact that different mathematical functions can be used to fit the epidemiological data relating incidence of skin cancer and the UV dose.[14] The exponential model is a log–linear relationship and can be written as

$$\ln R = \ln a + b\,U + e$$

where R is the skin cancer incidence, U the UV dose, a is a constant, e is an error term and b is the BAF. The power law model is a log–log relationship and can be written as

$$\ln R = \ln a + b \ln U + e$$

Using an exponential function, the value of BAF varies with latitude, being less far away from the equator.

A power-law relationship provides a constant value of BAF. For non-melanoma skin cancer, the BAF value is independent of age, dependent on sex, and higher for squamous cell carcinoma than for basal cell carcinoma. Table 2 shows BAF values for basal cell carcinoma and squamous cell carcinoma based on an analysis of the 1977–78 National Cancer Institute Survey using an exponential function.[15] Table 3 shows BAF values for non-melanoma skin cancer based on two separate surveys of skin cancer in the USA using both exponential and power-law functions.[14] The 1971–72 Third National Cancer Institute Survey yielded higher BAF values than the 1977–78 survey. Using a power-law relationship, the figures are 2·7 *vs* 1·9 for men and 2·2 *vs* 1·4 for women. Values for BAF for basal cell and squamous cell carcinomas are based on the later National Cancer Institute Survey, and the figures are approximately 3·0 for squamous cell carcinoma in both men and women, and for basal cell carcinoma 2·0 in men and 1·5 in women.

Values of BAF derived for melanoma show much greater variability and provide lower values for mortality than for incidence. The Third National Cancer Institute Survey, for example, produced values of 1·4–2·4 for incidence and 0·7–1·2 for mortality. These figures, however, should be regarded as the upper limit, and other studies have produced lower values for both incidence and mortality.[16] Overall, a BAF value of 0·7 for

Table 2. Biological amplification factor (BAF) values for basal cell carcinoma and squamous cell carcinoma (based on 1977–78 National Cancer Institute Survey[15])

Sex	Basal cell carcinoma	Squamous cell carcinoma
Men	1·32–2.59	2·08–4.09
Women	1·06–2.07	2·18–4.30

Table 3. Biological amplification factor (BAF) values for non-melanoma skin cancer (based on Fears and Scotto[14])

	BAF	
Sex	Power-law model	Exponential model
1977–78 National Cancer Institute Survey[15]		
Men	1·9	1·4–2·3
Women	1·4	1·1–1·8
1971–72 Third National Cancer Institute Survey		
Men	2·7	2·1–3·3
Women	2·2	1·7–2·7

mortality and 1·4 for incidence of melanoma seems reasonable.

In calculating the total amplification factor, it is necessary to multiply the values of OAF and BAF for each type of skin cancer. Since there is no experimental model for basal cell carcinoma or for malignant melanoma, it is necessary to rely upon the action spectrum derived for squamous cell carcinoma, and to assume that the OAF value is the same for all three types of skin cancer in humans. On this basis, and assuming no changes elsewhere, a 10% loss of total column ozone would be expected to increase melanoma mortality by approximately 10% and the incidence of melanoma by 20%, of basal cell carcinoma by 30% and of squamous cell carcinoma by almost 50%. Another way of expressing these predictions is to state that, if atmospheric ozone concentrations this century had been 10% less, the observed number of cancers in white populations would be increased by the above amounts. Although data for non-melanoma skin cancer are far from complete, this would probably mean an extra 150,000 cases of non-melanoma skin cancer annually in the USA, and perhaps 7000 in the UK. For melanoma, this would mean an extra 600 deaths annually in the USA, and perhaps 100 in the UK. Although most of these biological effects are avoidable, they should not be regarded as trivial. Dermatologists need to recognize the influence of environmental factors in determining the mortality and morbidity of their patients.

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British Journal of Dermatology (1992) 127, Supplement 41, 7–13.

The measurement of photodamage

R.MARKS AND C.EDWARDS

Department of Dermatology, University of Wales College of Medicine, Cardiff, UK

Summary The use of non-invasive and invasive techniques for the assessment of human photodamaged skin is reviewed. Physical changes during photodamage and its treatment are best scored using a visual analogue scale rather than a short, non-equal interval scale. Epidermal thickness can be measured by histometric methods but dermal thickness can be measured non-invasively using pulsed A-scan and B-scan ultrasound techniques. These approaches are not effective in detecting any changes due to photodamage. Mechanical properties of the dermis can be determined using either a static or a dynamic test mode. The authors have used extensometry to provide a measure of the laxity of skin. Replicas of the crow's foot areas have been taken before and after tretinoin treatment, and the replicas have been inspected by optical profilometry. Reductions of blood flow in photodamaged skin have been established using laser Doppler measurements, the effect being reversed by topical tretinoin. Invasive biochemical techniques have the disadvantage that they generally require large amounts of tissue. Cytochemical techniques, however, have shown increased glucose-6-phosphate dehydrogenase activity in the granular cell layer of patients with non-melanoma skin cancer, premalignant epidermal lesions, sun-damaged epidermis and artificially irradiated skin. This technique may provide an important model for the study of photodamage. It is concluded that there is no single method available to quantify the degenerative changes associated with photodamage and the effects of tretinoin.

There is no doubt that persistent exposure to the sun causes a multitude of clinically important degenerative changes in the various tissue compartments of skin. These include epidermal alterations which, after phases of hyperplasia and dysplasia, culminate in a range of neoplastic disorders. Melanocytes and Langerhans cells share in the epidermal 'distress' that characterizes chronic solar damage, and show changes in number and function. The dermis is also massively deranged by the characteristic morphological, biochemical and functional changes observed in the process that has come to be known as solar elastotic degeneration.

The existence of the general relationship between solar ultraviolet radiation (UVR) and the above tissue changes is widely accepted, but there are many specific questions that still require answers. In view of the enormous increase in both melanoma[1] and non-melanoma skin cancer,[2] and the accelerating decrease in the protective ozone layer,[3] answers to many of these questions are urgently required. Some of the questions that require research effort are as follows. Is the UVR dose–tissue effect relationship linear and, if it is, is it linear for all skin tissues? Is dose rate an important consideration, or is it only the cumulated UVR dose? What is the relative importance of UVA (long-wave UVR) and UVB (medium-wave UVR) in the different types of tissue damage? Do equal doses sustained at different ages have equally deleterious effects? Do sunscreens protect against any or all of the components of photodamage? Do topical retinoids and/or other agents reverse all the components of photodamage? All these questions require a quantitative approach—qualitative answers may provide important clues, but they cannot yield the information needed.

Having established that many questions need to be tackled, and that to do so adequately requires the use of measurement techniques, it has to be asked, what appropriate techniques exist now and what needs to be developed?

Techniques currently available

There are three important points that need to be made before reviewing the various methods. First, the technique chosen has to be appropriate to the question asked. There is not much point in measuring blood flow, for example, if the issue raised concerns epidermal dysplasia. Secondly, it has to be recognized that there is no such thing as a perfect technique, and that most studies

Correspondence: Professor R.Marks, Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK.

require the use of two or more methods to maximize the chances of producing a useful and interpretable group of data. Finally, and regardless of whether one chooses a clinical, histological, instrumental or biochemical test, the same stringent criteria have to be employed. Is the test mode sufficiently reproducible? Is it adequately sensitive for the purposes required? And is it sufficiently accurate with regard to reflecting the tissue alterations of interest?

Non-invasive methods

Non-invasive methods clearly have much to commend them in comparison with invasive techniques, but it is difficult to make them objective.

Clinical scoring techniques

Clinical scoring techniques are the simplest to perform, but among the most difficult to do well. The scoring of physical signs on short, non-equal interval scales (e.g. 0, no change; 1, slight improvement; 2, moderate improvement; 3, marked improvement) is usually insensitive and then, if the scale is expanded, is often not reproducible. Furthermore, the results of investigations using such scales are subject to misinterpretation as change at one end of the scale (i.e. improvement from grade 0 to grade 1) may not carry the same clinical significance as change at the other end of the scale (i.e. improvement from grade 2 to grade 3). In addition, the statistics of data obtained from these non-equal interval scales can be difficult to handle.

The authors' preferred option in the assessment of physical signs is to use 10-cm visual-analogue scales (Fig. 1) to 'score' the severity. These appear to be surprisingly reproducible, and are much more sensitive than grading scales. They have been employed to score the signs of photodamage during treatment with either 0·025% tretinoin cream or the bland, unmedicated vehicle in a double-blind treatment trial. Assessments were made at monthly intervals over a 3-month treatment schedule.[4] It was found that such signs as fine facial wrinkling and sallow yellowish discoloration improved at 8 or 12 weeks, and it appeared that the visual-analogue-scale scoring system made the evaluations considerably easier to complete.

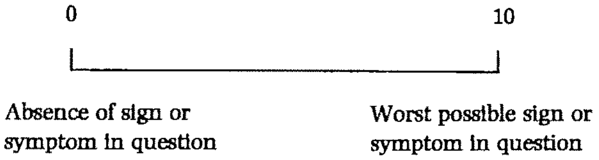
0
10
Absence of sign or symptom in question
Worst possible sign or symptom in question

Figure 1. A 10-cm visual-analogue scale.

Instrumental methods to assess physical signs

As the skin ages it becomes thinner,[5,6] and the ultrasound technique used to measure skin thickness non-invasively has proved very effective for this purpose. Pulsed A-scan ultrasound using transducers with centre frequencies of 15–20 MHz and B-scan methods have both proved suitable for this purpose. An A-scan instrument (Dermotronics Ltd, UK) has been utilized by Lever *et al.*[4] to measure skin thickness after treatment with tretinoin, as well as in a study comparing topical tretinoin with an abrasive preparation,[7] and it was found that there were small increases in the skin thicknesses after use of these treatments.

Of potentially greater importance than the determination of whole skin thickness is the measurement of the thickness (or other dimension) of other skin components. Measurements of the thickness of the band of solar elastosis in the papillary and upper dermis are clearly vital to any study of the potential of a drug to remove solar elastosis. As the disorganized structure of solar elastotic tissue is comparatively echolucent compared with the orientated fibrillar normal dermis, it can easily be distinguished (and measured) on the echo tracing. Edwards *et al.*[8] have shown that measurements of solar elastosis made in this way correspond quite closely to histological measurements. The B-scan imaging method has also been successfully used to detect the degree of solar elastosis present.[9]

Epidermal thickness also decreases with age in light-protected areas,[10] with the epidermis being irregularly thickened in some places and atrophic in others in areas that show chronic actinic damage,[11] while topical retinoids consistently increase the thickness of the epidermis.[12] Such information can be obtained histometrically, but the ability to measure epidermal thickness non-invasively would make the measurement a much more useful parameter. The pulsed A-scan ultrasound method can measure epidermal thickness, when it is considerably thickened as in psoriasis, with surprising accuracy (Fig. 2),[13] but as its vertical resolution is of the order of 50 μm—approximately the thickness of the normal mature epidermis—it is unlikely to be of use at its present stage of development in measuring the changes seen in photodamage.

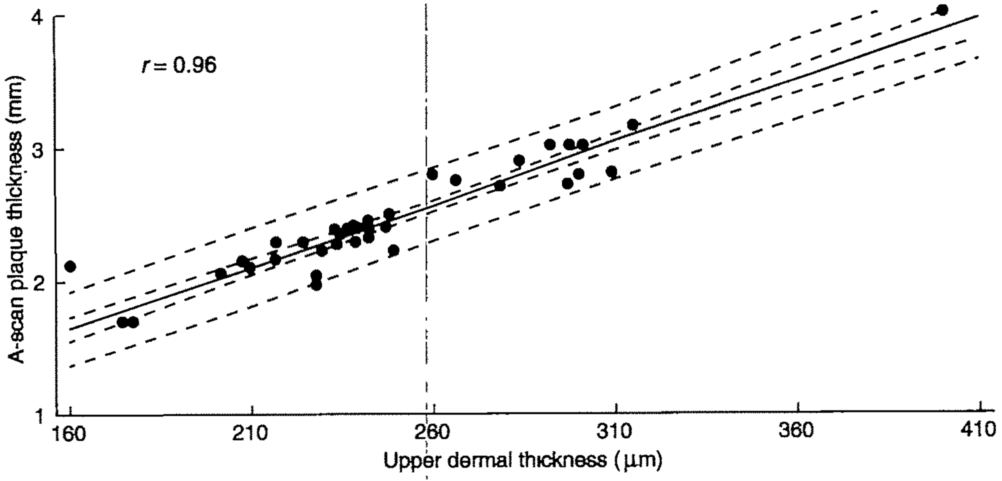

Figure 2. Scan plaque thickness *vs* upper dermal thickness by histology.

Mechanical properties of the dermis

Assessment of the mechanical properties and function of the dermis *in vivo* has proved to be a nightmare for bioengineers. The intimate interconnections of the different tissue compartments of skin, the anisotropy and time dependence of the mechanical properties, and the enormous regional variations in skin make mechanical measurements more difficult than any other type of skin measurement.

A variety of techniques have been devised to stimulate mechanically the skin surface in order to record the skin's response. Such tests are either static, with a single stimulus, or dynamic, with a rapidly repeated series of identical stimuli. The deforming mechanical stimuli used, with examples of the instruments devised and reports describing them are included in Table 1.[14–22] The fact that so many methods have been used suggests that no one technique is perfect. Each requires that the user is familiar with the particular instrument being used and

Table 1. Techniques for the measurement of mechanical properties of the dermis

Mechanical test mode	Instrument	Reference	Comment
Static:			
Torsion	Rotating disc and guard ring torque measurement	Agache *et al.*[14]	Increase in Young's modulus with age
Extensometry:			
Uni-axial	Uni-axial extensometer	Wijn *et al.*[15]	Increase in stiffness, loss of elasticity and loss of accommodation after stretching with age
Bi-axial	Bi-axial extensometer	Cook *et al.*[16]	
Compression	*In-vivo* indentometer	Dikstein and Hartshtark[17]	Results depend on skin moisture
Traction		Pierard *et al.*[18]	Photosclerosis increases extensibility and decreases elasticity
Suction	Suction cup	Cook *et al.*[16]	
Dynamic:			
Impact rebound	Ballistometer	Tosti *et al.*[19]	
Mechanical impedance	Electrodynamometer	Hargens[20]	Measures non-linear viscous properties
Elastic-wave propagation	Shear wave velocity	Potts *et al.*[21]	
Dynamic suction	Suction plus M-mode ultrasound measurement	Breitbart *et al.*[22]	

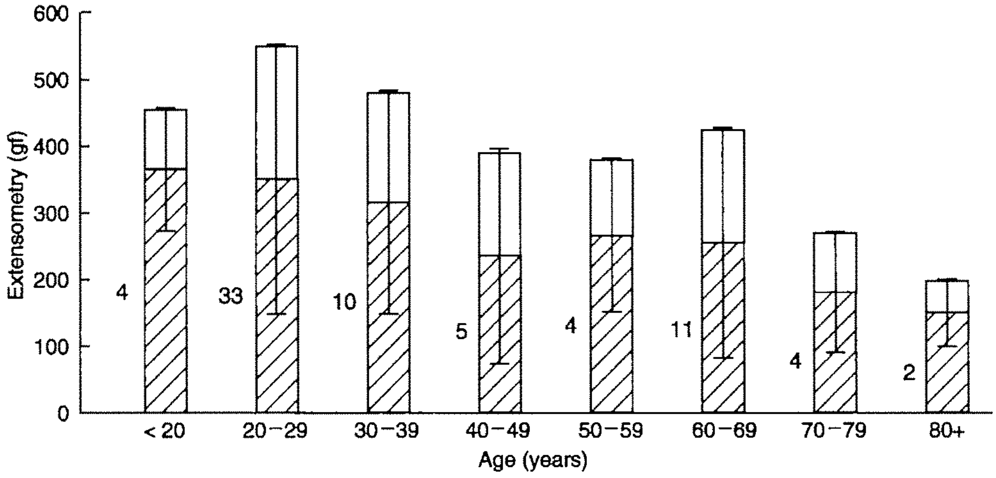

Figure 3. *In-vivo* extensometry changes with age in both sexes for the longitudinal arm axis.

its own inherent difficulties, including its reproducibility and sensitivity.

The authors' experience has been with a linear extensometer of the type described by Gunner *et al.*[23] It has been found that the peak force required to separate the feet attaching the device to the skin by 0·3 cm (initial separation 1 cm) decreased progressively with age (Fig. 3). As dermal collagen increases in stiffness with age, it is suspected that the device measures the overall slackness of skin with decreased fibrous protein per unit weight rather than the physical properties of dermal collagen. As dermal properties account for the bulk of the mechanical properties of the skin, most, but by no means all, of any change observed in extensometry is due to alterations in the dermis. This reduction in peak force required for separation of the skin attachments is presumably a reflection of the laxity of the skin structure, even though the altered collagen may itself be stiffer.

The regional mechanical function of skin is more important than the detailed physical properties of each of the skin components, although these must contribute to the function, and both are relevant to photodamage. The skin 'folds', 'droops' and 'jowls' of the elderly seem to be observed in chronically photodamaged subjects more than in the non-photodamaged, and such changes may ultimately be due to solar elastotic change.

Attempts are currently being made to monitor these changes with another device known as the 'Turgometer', which measures forces required to pinch up a fold of skin and pull it away from the surface.[24]

Skin surface profile

A major parameter of photodamage and of its improvement after treatment with topical retinoic acid has been the surface contour of the skin at the lateral angle of the eyes—the so-called 'crow's foot area'. In recent multicentre trials, important objective evidence of the effectiveness of topical tretinoin was obtained using optical profilometry.[25] In these studies, replicas were taken from the crow's feet areas of subjects before, during and after treatment with topical tretinoin and sent to a central location (G.Groves, Philadelphia, USA), where the replicas were examined and measured in an image-analysis device. This method of optical profilometry inspected the replicas in both 'east–west' and 'north–south' directions using standardized roughness parameters adapted for the particular task in hand.

Measurement of cutaneous blood flow

Another inevitable consequence of ageing is the reduction in the rate of blood flow to the skin, and an accompanying decline in the number and size of papillary capillaries. This process is accentuated in photodamaged skin, and may be one explanation for the alteration in skin colour noted in photodamaged areas.

Topical retinoic acid certainly increases the rate of skin blood flow, and patients appreciate the 'rosy glow' that results from retinoic acid preparations. Von Willebrand factor antigen increases in retinoic acid-treated skin, strongly suggesting an increase in the endothelial cell content of skin and implying angiogenesis.[26] The laser Doppler device measures the flux of red blood cell movement and is a good parameter of blood flow in the superficial part of the skin.[27] Several groups, including the authors', have used this device to track the increased skin blood flow that takes place after use of topical tretinoin.[7,28] The results are striking, and this is clearly a sensitive and convenient method.

Skin colour changes in chronically photodamaged areas, although the reasons for this are not yet clear. So far, the changes that take place have not been adequately quantified, and much more work is needed on the changing optical properties of skin in the elderly and ways in which these can be adequately documented.

Invasive techniques

Biochemical methods for the most part require too much tissue to allow serial measurements, and are not at present suitable as routine measures of photodamage. It may be possible, however, to adapt and 'miniaturize' biochemical estimates of elastic tissue to obtain an index of elastotic degenerative change. Work in this area is currently in progress, and hopefully will yield answers in the near future.

Quantitative cytochemical techniques have a little more to offer than biochemical tests, although they are often cumbersome and costly. The glucose-6-phosphate dehydrogenase (G6PDH) reaction in the granular cell layer of the epidermis has been a useful indicator of photodamage. By employing a microdensitometric technique to measure the amount of formazan reaction product throughout the thickness of the epidermis, it has been possible to detect significant increases in granular cell layer G6PDH activity in non-melanoma skin cancer, premalignant epidermal lesions, sun-damaged epidermis and artificially irradiated skin.[29,30] This is potentially an important model, but requires specialist knowledge and experience in the area.

The major changes in the dermis that take place during ageing and photodamage are both quantitative and qualitative. The dermis thins with age, as pointed out previously. It also alters biochemically and biophysically, but these changes are not easy to detect using histological methods. Profound alterations to the upper dermal connective tissue occur with ageing in the form of solar elastotic degenerative change. Several attempts have been made to quantify the amount of degenerative change present, but no entirely satisfactory method has evolved. A grading scale method has been employed,[31] as has a visual-analogue-scale scoring method by the authors' group (R.Marks, unpublished data). In addition, image-analysis methods have been used[32] but, because not only depth of elastotic change but also the density and horizontal extent of the degenerative change are important variables, the measurement is actually quite difficult. It is clearly important in relation to the action of tretinoin to be able to quantify accurately the degree of solar elastotic change. Double-blind clinical studies on the action of tretinoin that have included biopsies have not, as yet, identified any decrease in the amount of elastic staining material present.[4,7,33] When a suitable accurate and reproducible method is developed, long-term (> 6 months) studies of the effects of topical 0·05% tretinoin should be conducted to solve the paradox of there being marked clinical change unmatched by histological alterations.

It may, of course, not be the fibrous elements that change with topical tretinoin, but the interfibrillary ground substance, and hints of this have been noted qualitatively. To give form and substance to this possibility, however, its measurement will be necessary.

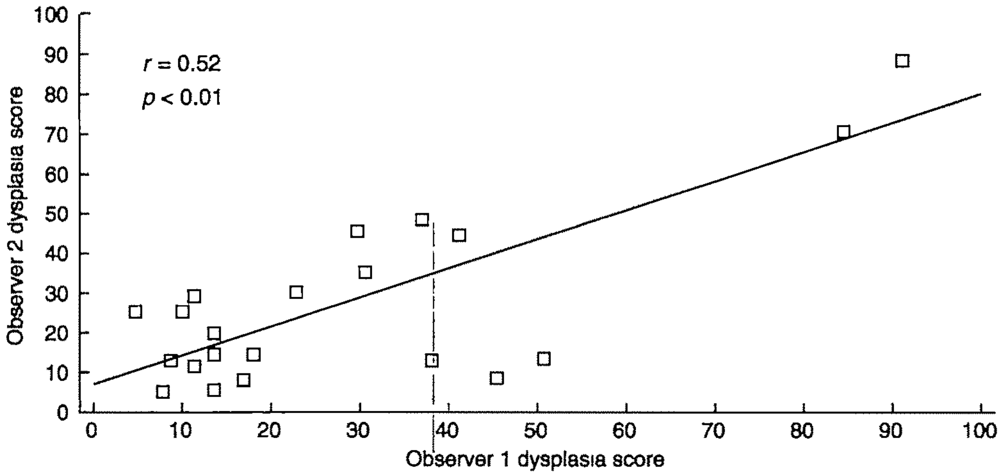

Figure 4. Inter-observer correlation of dysplasia scores of histological sections of biopsies using visual-analogue scales.

As noted previously, the epidermis thickens in tretinoin-treated skin, and does so irregularly in chronically sun-exposed skin. Measurements of these changes have proved useful and served as a model for the action of topical retinoid substances.[34] A further issue concerns the dysplasia consistently noted in photodamaged skin,[35] which presumably presages the development of non-melanoma skin cancer. Topical tretinoin decreases the number of solar keratoses present in a population,[36] but its potential for dealing with the generalized epidermal dysplasia of sun-damaged skin is as yet uncharacterized. The authors have attempted to quantify the degree of dysplasia present using image-analysis methods (Fig. 4),[37] but believe this approach is cumbersome and not suitable for routine purposes. The best method to date has been a visual-analogue-scale scoring technique which is at least reproducible between observers and within the same observer (Fig. 1),[38] but clearly this falls short of the ideal, and other methods are being explored.

Conclusion

As the population ages, the problems of ageing skin will increasingly occupy dermatological practice. In order to develop treatments and management strategies for dealing with these problems, the alterations that occur in the skin both with intrinsic ageing and with cumulated sun exposure need to be characterized and quantified. To this end it would seem sensible to devote more attention and resources to the development of appropriate methods for measurement of these changes, as current techniques are inadequate for this purpose.

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British Journal of Dermatology (1992) **127**, Supplement **41**, 14–20.

Retinoids and photodamage

B.A.GILCHREST

USDA Human Nutrition Research Center on Aging, Tufts University, and Department of Dermatology, Boston University School of Medicine, Boston, USA

Summary

Extensive well-controlled clinical studies performed over the past 5 years have demonstrated a consistent, dose-dependent, statistically significant improvement in the appearance of photodamaged skin after 3–6 months of daily treatment with topical 0·001–0.1% tretinoin cream. Clinical changes included decreases in surface roughness, irregular pigmentation, fine and coarse wrinkling, and sallowness. Actinic keratoses have also been reported to decrease in size and number. Blinded analysis of biopsies from more than 500 subjects showed that there was compaction of the stratum corneum, an increase in the number of granular layers, thickening of the epidermis and a decrease in epidermal melanin. There were no detectable histological changes in any dermal parameters. The specific cellular mechanisms by which retinoic acid (RA) exerts its beneficial effect on photodamaged skin are currently the subject of intensive investigation. It is well established that RA enters the nucleus where it binds to an RA receptor (RAR), and that the RA–RAR complex then binds to specific RA response elements in the DNA, modulating the expression of target genes. It is thus likely that RA improves at least some aspects of photoageing by modifying cellular differentiation programmes, as retinoids have been shown to do during embryogenesis, in malignantly transformed cells and in skin affected by certain dermatoses.

Over many decades, exposure to sun adversely affects the clinical and histological appearance of human skin as well as its function; a composite of changes commonly termed photoageing. Solar ultraviolet (UV) radiation also acts as an initiator and promoter of skin cancer, whose development is further facilitated by UV-induced local and systemic immunosuppression.[1] The impact of photoageing and photocarcinogenesis is rapidly increasing in developed nations throughout the world as a result of increased life span and hence larger populations of middle-aged and elderly individuals. Lifelong exposure to the sun and, most recently, evidence of a decrease in the protective ozone layer with a resultant increase in shortwave UV exposure are also contributory factors to the increased incidence. Skin cancer, the best studied and medically most significant consequence of habitual sun exposure, has indeed been described as an epidemic.[2] Preventive and therapeutic approaches to the problem of chronic photodamage are thus urgently needed.

The family of naturally occurring and synthetic compounds related to vitamin A (retinol), termed retinoids,[3] has been widely employed as therapeutic agents in recent decades. This article briefly reviews the extensive data suggesting that retinoids can improve at least certain aspects of chronic photodamage, and speculates on their relevant mechanism of action.

Treatment of photoageing

Most published data regarding the effect of retinoids on photoageing concern the use of topical tretinoin (all-*trans*-retinoic acid). The usefulness of tretinoin in reversing the effects of chronic UV irradiation was first demonstrated in the hairless mouse model in which, following an irradiation protocol capable of inducing severe elastosis, topical tretinoin therapy accelerated and enhanced the development of a dermal repair zone with newly synthesized collagen and elastin.[4] These experiments were, in turn, inspired by the anecdotal observation that topical tretinoin administered to middle-aged women for the treatment of acne incidentally improved the appearance of their photoaged facial skin.[5] In the first controlled trial of topical trentinoin therapy for human photoageing, 30 subjects were treated for 4 months with 0·1% tretinoin cream or vehicle control to both forearms and the face.[6] All subjects showed a statistically significant improvement in tretinoin-treated skin. Improvements were specifically noted in both fine

Correspondence: Barbara A.Gilchrest, MD, Department of Dermatology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, USA.

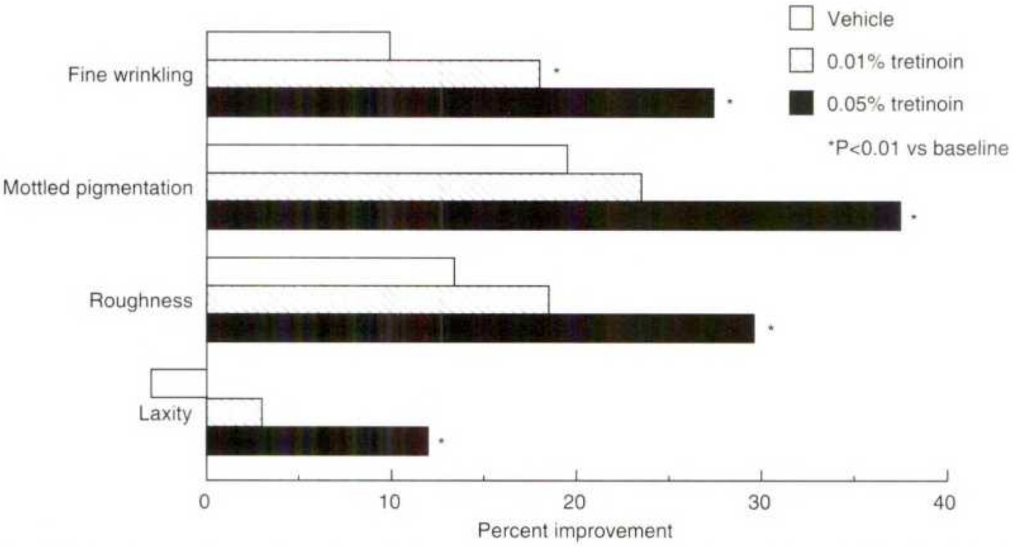

Figure 1. Percentage improvement in semi-quantitative 10-point scale score after treatment with 0·01% or 0·05% tretinoin, or vehicle emollient cream, for 24 weeks.

and coarse wrinkling, roughness, irregular pigmentation and sallow colour of the skin. Skin biopsies obtained at the conclusion of therapy demonstrated an altered stratum corneum morphology and increased thickness of the epidermis and granular cell layer compared with vehicle-treated control areas. These findings have since been confirmed by many other investigators in double-blind, vehicle-controlled trials involving more than 700 subjects and lower concentrations of topical tretinoin.[7–10]

Representative of this experience is a double-blind multicentre study conducted, in part, at Boston University School of Medicine.[9] After 6 months of daily application of 0·05% or 0·01% tretinoin, or emollient vehicle cream alone, a modest but highly statistically significant dose-dependent improvement was noted in the overall severity of photoageing and specifically in fine wrinkling, mottled hyperpigmentation, roughness and laxity (Fig. 1). Subject self-assessment was even more positive. Mild cutaneous adverse reactions were reported by 78% of those using 0·01% tretinoin cream, 90% of subjects using 0·05% tretinoin cream and 60% of those using emollient vehicle cream alone, suggesting a low threshold to reporting irritation in this trial. The

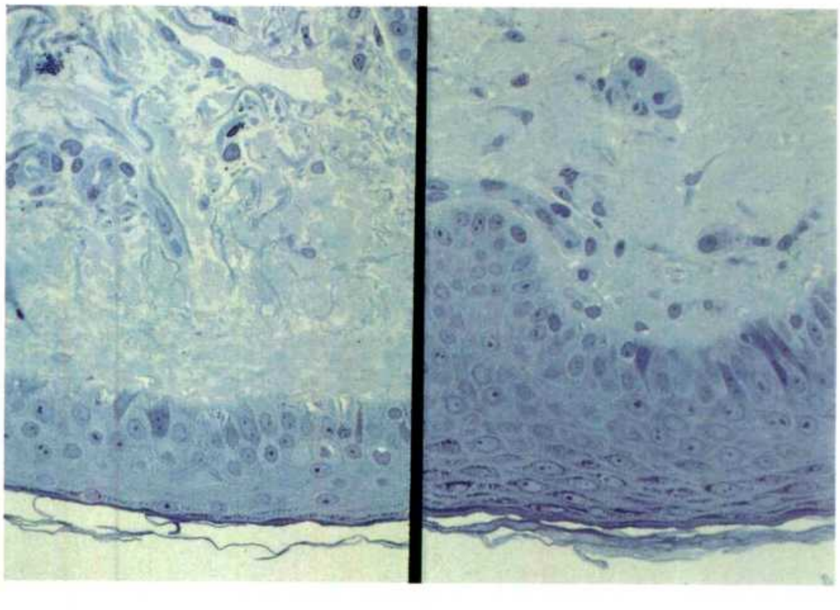

Figure 2. Histological appearance of facial skin before (left panel) and after (right panel) 6 months of treatment with 0·05% tretinoin cream. There are marked increases in epidermal thickness and number of granular cell layers, and the pretreatment basketweave pattern in the stratum corneum is altered to a compact pattern (toluidene blue, original magnification × 13,000, courtesy of J.Bhawan, M.D.).

Table 1. Histological effects of topical tretinoin and vehicle cream on photoaged skin

Treatment	Percentage change from baseline to 24 weeks* Epidermal thickness	Number of granular cell layers	Epidermal melanin content	Biopsies with compact stratum corneum
Vehicle cream	12	16	−31	32
0·001% tretinoin	16	16	−28	44
0·01% tretinoin	28	37	−48	57
0 05% tretinoin	33	54	−61	68

* All percentages (with the exception of the 0·001% tretinoin treatment group, studied in only one multicentre trial) are weighted composite figures from two separately conducted multicentre trials of the same tretinoin preparations, using the same study protocol.[10]

great majority of subjects therefore experienced mild to moderate improvement in their photoageing within 6 months of topical tretinoin therapy, with greater improvement observed at the higher tretinoin dose. Users of vehicle alone also experienced slight improvement compared to their baseline status, suggesting an intrinsic repair capacity for sun-damaged skin that is detectable after only 6 months of rational skin care and sun avoidance.

The histological changes accompanying successful tretinoin therapy of photoageing have also been extensively studied. In the most comprehensive study to date, facial biopsies were obtained before and after 6 months' treatment with 0·001%, 0·01% or 0·05% tretinoin cream, or vehicle alone, from 533 subjects enrolled in two double-blind, randomized clinical studies performed at a total of eight universities.[10] The biopsies were coded and analysed in a blind manner using both semi-quantitative conventional histological scoring and computerized image analysis. Consistent with clinical results in the same subjects and with earlier less extensive histological analyses, four statistically significant dose-dependent differences from vehicle were found in the tretinoin-treated epidermis: increased overall thickness; increased granular layer thickness; decreased melanin content; and stratum corneum compaction (Fig. 2, Table 1). There were no detectable changes in any of the dermal parameters examined: thickness of papillary dermis (presumptive repair zone); elastin and collagen content; or severity of perivascular inflammation. As in the clinical studies, the vehicle cream and/or the accompanying sun avoidance and skin-care regimen also produced similar changes from baseline evaluation within the 6-month study, although the changes were statistically less significant than those observed with either 0·01% or 0·05% tretinoin cream.

Whether the rather striking histological changes are causally related to the clinical improvements observed in the same subjects is unknown at present. With the exception of pigmentary changes, there is a poor statistical correlation in individual subjects,[10] possibly because the biopsy specimens were obtained from one 2-mm site in the crow's foot area, whereas clinical assessments were made globally for the entire face. It is also possible that the observed histological changes are simply markers for retinoid effects on the epidermis, rather than being related to the clinical improvement. This possibility is made more likely by the fact that, despite continued clinical improvement, biopsies obtained after 12 months of therapy in the same subjects showed partial or complete regression of the epidermal changes, again with the exception of decreased epidermal pigment content, which persisted.[11] Preliminary observations at the immunofluorescent and/or electron microscopic level have revealed subtle dermal changes that may, however, ultimately prove to be more clinically relevant. Tretinoin-treated human dermis appears to contain increased anchoring fibrils[12,13] and animal studies have revealed, in addition, deposition of new papillary dermal collagen and elastin.[4] These changes are intuitively more likely than those noted in the epidermis to explain the decreased wrinkling in tretinoin-treated skin. Chronic tretinoin-induced inflammation leading to oedema and, hence, effacement of wrinkles has been excluded as a possible explanation.[10]

Prevention and treatment of cutaneous malignancy

Retinoids have long been studied as possible chemotherapeutic and chemopreventive agents for cancer, including skin cancer. Partial or complete regression of basal cell carcinomas, keratoacanthomas and even metastatic melanoma has been reported in small patient series.[14–20] Oral isotretinoin has more recently been reported to decrease the incidence of new skin cancers in five patients with xeroderma pigmentosum treated over a 2-year period.[21] As in earlier studies, however, new tumour formation resumed promptly upon discontinuation of the drug. It is interesting to note that high oral doses of the proretinoid *β*-carotene failed to decrease the incidence of new non-melanoma skin cancers in a large, carefully controlled trial of high-risk patients treated for 5 years.[22]

A more practical clinical use of retinoids in the treatment of invasive malignancies is their ability to cause regression of premalignant skin lesions, such as actinic keratoses. Rapid clearance of actinic keratoses by oral administration of etretinate was shown in one well-controlled crossover study of 44 patients.[23] A large, as yet unpublished, multicentre study similarly found that topical 0·05% tretinoin cream applied once or twice daily decreased the number and size of facial actinic keratoses by approximately 50% after 6–15 months. This effect was statistically superior to vehicle cream application and sun avoidance alone, although the latter approaches were also associated with improvement.[24] These findings are consistent with the well-documented role of oral isotretinoin in preventing the progression of oral leucoplakia to squamous cell carcinoma of the oral cavity,[25] and in preventing development of second primary head and neck tumours in patients already treated for squamous cell carcinoma involving the oral cavity, pharynx or larynx.[26]

Mechanism of retinoid effects

Retinoic acid (RA), the oxidative metabolite of retinol, is widely assumed to mediate physiological retinoid effects in most tissues,[27–30] although derivative compounds may also be active.[30,31] Other therapeutically administered retinoids may act directly or may undergo conversion to RA. Complex regulatory mechanisms involving the intestine, liver and circulatory compartment ensure that the often large and unpredictable quantities of dietary retinoids and proretinoids do not undermine cellular RA homeostasis.[32]

Once retinoids reach the target cell following ingestion, systemic administration or topical application, additional regulatory mechanisms act in as yet poorly understood ways to modulate the ultimate cellular response (Table 2). The retinoid is first transported across the cell membrane either bound to a specific membrane receptor or via non-receptor-mediated endocytosis.[31] Within the cell cytoplasm, the active retinoid, presumably RA in most instances, binds to a cellular RA binding protein (CRABP), of which at least two distinct types are now recognized. Postulated roles for the CRABP include transport of RA to the nucleus,[33,34] thus promoting the ultimate effects of RA on cell behaviour, and reduction in the level of free RA available to reach the nucleus,[35] thus reducing the effects of RA on cell behaviour. Conceptually, different CRABPs may serve different roles at different times. The two recognized CRABPs vary markedly in their RA binding affinity and in their relative expression in different cell types.[36,37] Work at the protein level has demonstrated widely

Table 2. Speculative mechanisms for regulation of retinoid effects in the skin

Level of regulation	Possible target parameter(s)	Known modulators
Retinoid uptake	Ligand-specific membrane receptors	None
	Endocytosis	
Cellular retinoic acid-binding protein (CRABP)	Total CRABP protein level	Cell type Stage of embryonic development
	Relative abundance of CRABP I *vs* CRABP II	Degree of differentiation Disease states Retinoic acid, ageing and photoageing
Nuclear retinoic acid receptors	Total RAR protein level	Cell type Stage of embryonic development
	Relative abundance of different receptor proteins	Retinoic acid exposure
Retinoic acid-responsive genes	Baseline level of gene transcription Presence of other transacting factors	None

varying concentrations of CRABP in different tissues, at different stages of embryological development, in specific disease states and in response to retinoid deficiency or retinoid therapy.[38–46] The recent cloning of CRABP I and CRABP II complementary DNAs from several species, including man,[46–51] should greatly facilitate the exploration of CRABP regulation in response to various physiological and pathological stimuli and, in turn, should lead to an appreciation of the complex role of CRABPs in modulating retinoid effects at the cellular level.

A major breakthrough in understanding retinoid effects on cells has been the identification of specific high-affinity RA receptor (RAR) genes. These gene products are part of a large family of nuclear receptors for steroid and peptide hormones that act as ligand-inducible transcriptional enhancer factors.[52–58] Very elegant studies in many laboratories have demonstrated that RA, bound to RAR, binds directly to RA response elements in DNA and then modulates transcription of specific genes. At least five distinct RARs exist and these receptors provide a possible mechanism, depending on their relative abundance, for the different effects of RA in different cell types, in normal *vs* diseased or transformed cells and at different times of embryonic or postnatal development. The presence of other transacting factors, such as corticosteroids or thyroid hormone, also modifies the interaction of the RA–RAR complex with target genes.[59,60] Preferential transfer of RA from a specific CRABP species to a specific RAR species may provide a further level of regulation for the biological effects of retinoids.

Our understanding of the regulatory pathways relevant to retinoid effects on human skin is evolving rapidly. The activity of CRABP has been reported to vary between keratinocytes (higher) and fibroblasts (lower) in neonatal skin,[38] to be increased in lesional skin of psoriasis[39] and to be altered by topical or systemic retinoid therapy.[40] *In vitro*, terminal differentiation of keratinocytes is accompanied by increased CRABP concentrations.[41] Nuclear RARs have been studied primarily in mouse skin,[55] but recent work confirms their presence in human skin and cultured cells[56,61] and strongly suggests that RAR mediate RA effects on gene expression in skin.

The well-documented ability of RA–RAR complexes to modulate the expression of specific genes within specific cell types thus provides a plausible mechanism for the clinically observed effects of retinoids on photoageing and photocarcinogenesis. To date, physiological concentrations of RA have been reported to induce only interleukin-1β messenger RNA in cultured skin-derived cells (keratinocytes) over a time course that suggests transcriptional regulation.[61] Photocarcinogenesis, however, is known to involve aberrant gene expression. Work with malignantly transformed cells both *in vivo* and *in vitro* has demonstrated the ability of RA to modify the malignant phenotype, at least in part, by promoting differentiation through RAR interactions with the genome.[27,62] Photoageing is a far less well understood process, but recent studies suggest that certain genes, centrally involved in growth and differentiation, are abnormally regulated in keratinocytes derived from habitually sun-exposed (photoaged) skin, as compared to sun-protected skin.[63,64] It appears likely, therefore, that retinoids produce their beneficial effects on photoageing and photocarcinogenesis through modulation of cellular differentiation programmes involved in the pathogenesis of these disorders.

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British Journal of Dermatology (1992) 127, Supplement 41, 21–24.

Mechanisms of action of retinoic acid in skin repair

C.E.M.GRIFFITHS, G.J.FISHER, L.J.FINKEL AND J.J.VOORHEES
Dermatopharmacology Unit, Department of Dermatology, University of Michigan Medical Center, Ann Arbor, Michigan, USA

Summary The ability of topically applied retinoic acid to improve photoaged skin has stimulated research interest into its mechanism of action. Currently available assay systems are either *in-vitro* or mouse models, neither of which are truly representative of the *in-vivo* situation in man. Another drawback is that skin biopsies available from studies using retinoic acid to treat photoageing are of insufficient size to accomplish biochemical and molecular assays. In order to address these problems, a 4-day *in-vivo* retinoid assay has been developed which serves as a good predictive model for the chronic effects of retinoic acid on skin and helps to characterize the mechanism of action of retinoic acid.

Cutaneous photodamage or extrinsic ageing occurs as a consequence of prolonged exposure to ultraviolet (UV) and infra-red radiation, and is clinically manifested by wrinkles, dyspigmentation and sallowness.[1] The epidermis is initially thickened, progressing eventually to atrophy, and associated with keratinocyte atypia. Dermal changes include loss of collagen and increased elastotic material in the reticular dermis.[2] Work by Kligman,[3] using the hairless mouse model, has conclusively shown that topical all-*trans* retinoic acid (RA) can repair the cutaneous changes induced by UV radiation. These salient observations led to the initial investigations into the use of RA in the treatment of extrinsic ageing.[4]

Large, double-blind, vehicle-controlled studies lasting for periods of 4–22 months have provided incontrovertible evidence that topical RA (tretinoin) can efface at least some of the features of extrinsic ageing: namely, fine wrinkles, surface roughness and actinic dyspigmentation.[5–8] Clinical improvement is accompanied by striking changes in epidermal histology, i.e. stratum corneum compaction, spongiosis, thickened granular cell layer, increased epidermal thickness, increased basal keratinocyte mitotic index and deposition of epidermal mucin. Dermal changes are less obvious, although at the ultrastructural level there is a significant increase in the number of epidermal–dermal anchoring fibrils after tretinoin treatment for 4 months.[9]

The success of tretinoin for the treatment of photoageing has prompted two developments: a search for other so-called 'skin repair agents', and an intensification of research into the mechanism of action of tretinoin. In order to study the *in-vivo* effects of topical RA, an animal model such as the hairless mouse or rhino mouse[10] or, preferably, a human model can be used. The problems of using biopsy material from photoageing studies in man are twofold. First, in order to facilitate molecular and biochemical assays, large quantities of tissue are required; this is an impractical consideration from the face or forearms. Secondly, the long time period involved in clinical studies is also a rate-limiting factor. For these reasons, an attempt has been made to produce a short-term *in-vivo* human retinoid assay which can supply large quantities of tissue for the investigation and characterization of retinoid effects in the skin.

Development of a human retinoid assay

One of the most characteristic and early effects of the topical application of RA is the appearance of an erythematous, scaling and irritant response, usually within the first 2 weeks of use. Over time, this 'retinoid reaction' resolves and is only a problem to subjects during spells of cold, dry weather. It has been debated whether or not the retinoid reaction, a form of dermatitis, is a necessary component of RA's efficacy as a 'skin repair agent', i.e. is this purely an irritant response or is RA imbued with a more specific mechanism of action? The discovery of the nuclear retinoic acid receptor family (a member of the steroid, thyroid hormone superfamily of receptors) has provided compelling evidence for a specificity of action for RA.[11–13] These retinoic acid receptors act as ligand-inducible transcription enhancers, of which the γ-form is the best candidate for RA binding in skin.[14] In an attempt to answer these questions, a reproducible, short-term retinoid reaction in

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Correspondence: Dr C.E.M.Griffiths, Department of Dermatology, University of Michigan Medical Center, 1910 Taubman Center, 1500 East Medical Center Drive, Ann Arbor, Michigan 48109–0314, USA.

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humans has been developed, and this is believed to be a good model for studying the mechanism(s) of action of RA.[15]

The model involves the application of approximately 500 mg 0·1% tretinoin (RA) cream to a 27-cm^2 area of the buttock skin of normal volunteers. The cream is occluded using Saran Wrap secured with surgical tape for 4 days. This technique gives a very reproducible erythematous reaction with 0·1% tretinoin cream that contrasts with an absence of reaction following the use of the vehicle cream (Fig. 1). Histological examination delineates marked differences between tretinoin- and vehicle-treated skin using this assay. Buttock skin treated with tretinoin shows histological changes in the epidermis that are almost indistinguishable from those observed after 4 months' treatment of photodamaged skin. In comparison to vehicle-treated skin, tretinoin treatment produces a 70% increase in epidermal thickness, stratum corneum compaction, increased number of mitotic figures (3·8/10 high-power fields compared with 0·1/10 high-power fields for vehicle), spongiosis and a thickened granular cell layer (Fig. 2). The epidermal spongiosis is associated with mucin deposition as demonstrated by colloidal iron staining. All these features are identical to those seen in long-term tretinoin treatment. A consequence of the short time period is

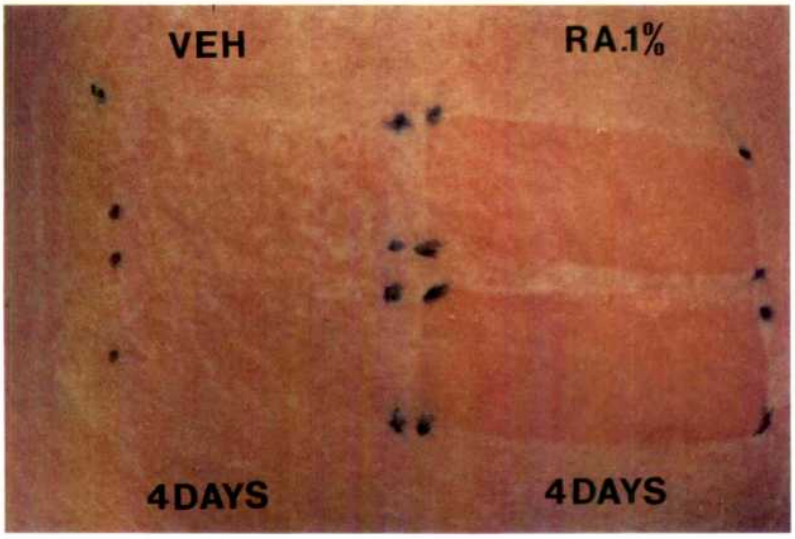

Figure 1. Erythema induced by 4 days of occlusion with 0·1% tretinoin cream (RA.1%), as compared with no reaction with vehicle cream (VEH).

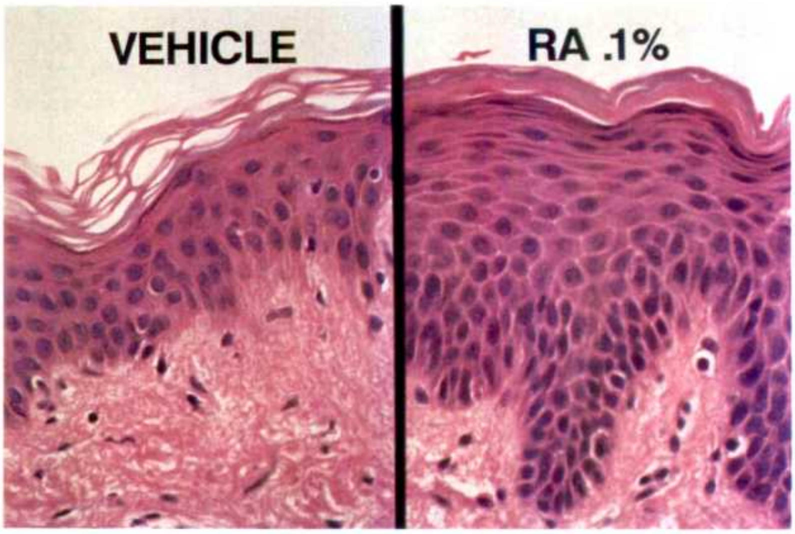

Figure 2. Histology of 4-day occlusion with vehicle cream (veh) and 0·1% tretinoin cream (RA). Note the increase in epidermal thickness, thickening of the granular cell layer and spongiosis in the tretinoin-treated skin (haematoxylin and eosin × 305).

the paucity of dermal changes (although these are not readily apparent even after 4 months' treatment), the most consistent dermal feature being the presence of a sparse perivascular inflammatory infiltrate. At the immunohistological level, it has been shown that the majority of perivascular lymphocytes are of the CD4 subset and are activated as evidenced by the expression of HLA-DR. There is a modest reduction in epidermal Langerhans cells in tretinoin-treated patients compared with vehicle-treated patients, with an increased prominence of dermal dendrocytes within the papillary dermis. Indicative of a direct effect of tretinoin on the keratinocyte is the focal expression of keratinocyte ICAM-1 occurring in the absence of an intra-epidermal inflammatory infiltrate.[14]

The patch test model lends itself to the study of the biochemical effects of tretinoin on skin, as larger keratome biopsies can be removed for enzyme assays, high-performance liquid chromatography and molecular analysis. Using this system, the retinoid response has been further characterized with regard to the inflammatory mediators involved.

From previous work on psoriasis, it is known that the phospholipase C/protein kinase C signal transduction pathway is chronically activated in this disorder.[16] There are certain similarities between psoriasis and the retinoid reaction, particularly the hyperproliferative component. The activities of constituents of this signal transduction pathway, namely phosphoinositide-specific phospholipase C, protein kinase C and the second messengers 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3), were determined from keratome biopsies. Phospholipase C was increased 2·5-fold in the tretinoin-treated compared with vehicle-treated skin, with a concomitant 1·6-fold increase in DAG concentrations. There was no difference in the IP_3 content of tretinoin-treated and vehicle-treated skin. Protein kinase C activity in tretinoin-treated skin was reduced by one-third compared with vehicle—a finding consistent with chronic activation and subsequent down-regulation of this enzyme. A similar scenario is observed in psoriasis.

Other inflammatory mediators were also investigated: the metabolites of the arachidonic acid cascade (eicosanoids); and interleukins-1α and 1β. No significant differences in eicosanoid concentrations and interleukin-1α were discerned between tretinoin- and vehicle-treated skin. Interleukin-1β messenger RNA concentrations were decreased by up to 70% in tretinoin-treated compared with vehicle- treated skin, and interleukin-1β protein concentrations were decreased by 12%. This observation of relatively little change in inflammatory mediators is consistent with the finding of a sparse inflammatory infiltrate and the inference of a scarcity of chemoattractants.

The similarities between psoriatic skin and tretinoin-treated skin prompted an investigation of the proliferation and differentiation pathways in the patch test model and a comparison with the *in-vitro* situation with cultured keratinocytes. The data provide additional evidence for an apparent paradox between *in-vivo* and *in-vitro* effects of tretinoin.

Keratinocyte-specific transglutaminase K is responsible for cross-linking the substrate proteins, involucrin and loricrin, to form epidermal cross-linked envelopes, which are a necessary component of a cutaneous barrier. In psoriasis, the activity of transglutaminase K is greatly increased compared with that observed in normal skin.[17] The immunoreactivity of transglutaminase K and involucrin in normal skin is confined to a narrow, sub-stratum corneal band. By contrast, in psoriasis, there is precocious expression of both enzyme and substrate throughout the suprabasal layers of the epidermis.[18] RA *in vitro* inhibits both enzyme activity and immunoreactivity of transglutaminase K, and thus one would predict that tretinoin down-regulates this enzyme *in vivo*. This is not the case because at 4 days in the patch test, both enzyme activity and immunoreactivity are increased, as is the immunoreactivity of involucrin.[19] Transglutaminase and involucrin immunoreactivity are also increased after chronic 4-month use of topical tretinoin for photoageing.[20] The differentiation markers, keratins 1 and 10, are suppressed by tretinoin in culture.[21] However, as for transglutaminase K, there is no inhibition in the 4-day model; a situation similar to that seen at 4 months.[19]

These experiments emphasize the importance of an *in-vivo* model, and suggest that, although tretinoin exerts some of its *in-vivo* effects directly via the keratinocytes, the interaction with the milieu of cutaneous cytokines and growth factors, coupled with the influence of the dermis, is a far more important determinant of the outcome.

Specificity of the model

A recurrent criticism of clinical research performed with topical tretinoin is that of the specificity of the response. Is the reparative action peculiar to tretinoin, or is it a consequence of chronic local irritation? A study by Marks *et al.*[22] compared the efficacy of a skin abrasive, Brasivol®, with that of topical 0·05% tretinoin cream on photodamaged forearms. They reported no significant epidermal histological differences between the two agents after 8 weeks of treatment, implying that the effects of tretinoin may be very similar to those of local irritation.

In an attempt to address this issue, a well-characterized irritant, sodium lauryl sulphate (SLS), was used in the patch test assay. A 2% concentration of SLS compounded in vehicle cream produced a consistent 4-day erythema of a very similar intensity to that produced by 0·1% tretinoin cream. Histological changes induced by 2% SLS were indistinguishable from those produced by 0·1% tretinoin at 4 days, the only difference being that tretinoin produced a greater increase in epidermal mucin than did SLS.[23] Recent work using the patch test for shorter time periods has helped to differentiate further the SLS reaction from that produced by tretinoin. At 1–2 days, tretinoin produced a significant increase in spongiosis, whereas SLS-treated skin showed no increase in spongiosis compared to the vehicle. This occurred despite the fact that SLS produced an erythema at 2 days (C.E.M.Griffiths, unpublished observation). It appears, therefore, that although SLS, and probably other irritants as well, can produce an epidermal histological picture very similar to that of tretinoin, the mechanism of action is different. Most probably, tretinoin exerts its effects via direct interface with retinoic acid receptors, whereas SLS works through other non-receptor pathways to produce similar results at 4 days.

Conclusions

The 4-day occlusion model has proved to be a reproducible assay for the study of the retinoid response in human skin. Although many of the observed changes are not specific for tretinoin, it would appear that at early time points, i.e. from 4 to 24 h, tretinoin-specific events are involved in this reaction. By contrast with *in-vitro* work, the short-term model also appears to be a good predictor of the epidermal changes observed with chronic use of tretinoin to treat photoageing and may be able to discriminate between active and inactive agents for this indication.

Acknowledgments

This study was supported in part by grants from the R.W.Johnson Pharmaceutical Research Institute, Raritan, NJ, and the Babcock Dermatologic Endowment, Ann Arbor, Michigan.

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British Journal of Dermatology (1992) **127**, Supplement 41, 25–30.

Ageing and photoageing of the skin: observations at the cellular and molecular level

B.A.GILCHREST AND M.YAAR
USDA Human Nutrition Research Center on Aging at Tufts University, and Department of Dermatology, Boston University School of Medicine, Boston, MA, USA

Summary It is now well established that ageing occurs at the level of individual cells in the skin and other organ systems. Changes in cell behaviour, protein production and gene expression in response to standardized stimuli are readily observed in cultured cells derived from young *vs* old donors and from photoaged *vs* sun-protected body sites. Whether these changes are best viewed as a cause or a consequence of ageing cannot be determined at present. Nevertheless, available data now provide cellular and molecular correlates for the well-known differences in clinical responsiveness between newborn, adult and photoaged skin. From this basis, it will hopefully be possible to develop a more comprehensive understanding of cutaneous ageing processes.

Ageing is accompanied by a progressive reduction in organ function and increased vulnerability to disease. In the skin in particular, many age-associated functional decrements and pathologies result from cumulative environmental insults in combination with intrinsic ageing.

Chronological or intrinsic skin ageing comprises the clinical, histological and physiological changes observed in sun-protected skin of most older individuals. The physiological changes include a decrease in all of the following: rate of epidermal turnover; rate of re-epithelialization after wound healing; clearance of chemical substances from the dermis; sensory perception; mechanical protection; immune responsiveness; vascular reactivity; thermoregulation; sweat and sebum production; and the capacity for vitamin D synthesis.[1]

Photoageing consists of those changes that occur in habitually sun-exposed older skin. These changes are sometimes an exaggeration of those seen in chronologically aged skin, but there are many striking differences as well. Photoaged skin is clinically characterized by a coarse or pebbly quality termed 'elastosis', irregular pigmentation, roughness, telangiectasia, atrophy, fine and coarse wrinkling, and a variety of benign and malignant neoplasms.[1,2] Functional decrements are widely believed to be uniformly more severe than in sun-protected skin of the same individual, although supporting experimental data are only available for immune responsiveness.[2]

In-vitro models of cellular ageing

Studies of cellular ageing were first undertaken by Alexis Carrel nearly a century ago.[3] Using newly developed cell-culture techniques, he questioned whether ageing occurred within individual somatic cells or only at the level of the whole organism. He erroneously concluded, because of limitations in experimental systems then available, that individual cells did not age and that, removed from the body, cells were immortal.[3,4] This belief was not seriously questioned until the work of Hayflick in the 1960s. His seminal studies demonstrated that human fetal lung fibroblasts had a finite, reproducible life span and that, after 50–60 cumulative population doublings, they would cease to divide under culture conditions that initially supported exponential growth.[5,6] He also noted that cells derived from older donors had a shorter culture lifespan.[7] These results were soon confirmed by others, who also determined in adults an inverse relationship between dermal fibroblast culture lifespan and donor age, and a shortened culture lifespan for fibroblasts derived from patients with premature ageing syndromes compared to age-matched controls.[8–15] The general applicability of Hayflick's fibroblast findings (finite culture lifespan and decreased proliferative capacity at late passage or with advanced donor age) has since been confirmed in all cell types examined to date, including skin-derived keratinocytes[1,16–18] and melanocytes.[19–21]

From the above observations, Hayflick and others concluded that differences between early passage- and late passage-cultured cells (*in-vitro* ageing) were closely

Correspondence: Barbara A.Gilchrest, MD, Department of Dermatology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, USA

related to differences between cells in tissues of young adults compared to old adults, and could thus explain age-associated functional losses of medical significance. The *in-vitro* model of cellular ageing specifically assumes that the same events that are responsible for *in-vitro* senescence also occur *in vivo* and are responsible for clinical manifestations of ageing including, but not necessarily limited to, decreased cellular proliferative capacity as observed, for example, in slow wound healing. In view of the heavy reliance on this model system by gerontologists,[22] relatively few data support this central tenet. Some investigators, including the authors, have therefore preferred to work with an *in-vitro* model of cellular ageing that compares cells of young adults with those of old adult donors at the earliest passage in which sufficient cells are available for study, in the belief that this comparison is more likely to reflect true age-associated changes. Direct comparisons between the two models of cellular ageing have shown some differences between cells 'aged' *in vitro vs in vivo*,[23] and the validity of both models remains a subject of discussion.

Manifestations of intrinsic ageing in cultured skin-derived cells

In-vitro studies have demonstrated that the chronological age of the donor is reflected in the behaviour of cultured skin-derived cells. These differences are most striking between newborn and adult donors, but can also be detected between young adult and old adult donors, despite large inter-donor variability. This fact is important, given the justifiable concern that differences between the newborn and adults may reflect development rather than ageing.

Initial studies demonstrated that dermal fibroblasts from donors of different ages progressively lost their responsiveness to exogenous mitogens.[24–26] The marked decline in growth potential of aged fibroblasts may reflect one or more of the following: a loss of high-affinity receptors to specific growth factors, as was shown for the epidermal growth factor receptor;[30] failure of the receptor to autophosphorylate after ligand binding,[31,32] although the extent to which this occurs in intact cells is unknown;[33–35] or decreased capacity of post-receptor signal transduction pathways, as has been recently suggested for keratinocytes.[36] Keratinocytes have also been found to show an age-associated decrease in both mitogenic responsiveness[26,34] and colony-forming potential.[16,37,38] Cellular release of factors capable of stimulating cells of the same type (autocrine growth factors) or neighbouring cells (paracrine growth factors) also declines with donor age.[29,39] Adult donor keratinocyte cultures produced significantly ($P<0{\cdot}01$) less epidermal cell-derived thymocyte activating factor (similar or identical to interleukin-1β) per cell than newborn donor keratinocytes after confluent culture for 72 h in serum-free, hormone-supplemented medium. This finding may have both immunological and proliferative implications for skin ageing.[39] Overall, the effect of increasing donor age on response to both exogenous and endogenous mitogens is striking (Fig. 1).

The controls for epidermal cell proliferation *in vivo* are largely unknown. In normal skin, however, approximately 95% of cells in the germinative basal layer do not cycle regularly, suggesting that growth inhibitors are present. An interferon-like molecule in the basal layer of the epidermis has been identified that may play a physiological role in inhibiting cellular proliferation.[40]

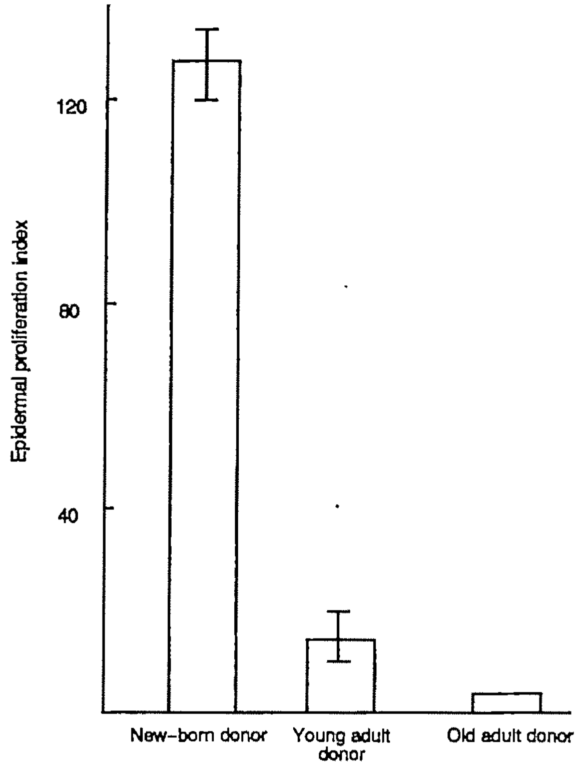

Figure 1. Influence of age on the epidermal proliferation index, defined as the product of cell yields for 10 donor cultures in each age group assayed for responsiveness to KGF, EGF and undefined mitogens present in keratinocyte-conditioned medium. Keratinocyte growth factor is an activity in bovine hypothalamus consisting, in part, of inositol[27] and, in part, of a protein growth factor closely related to fibroblast growth factor.[28,29] (Reproduced with permission from Stanulis-Praeger and Gilchrest.[26])

Preliminary data suggest that epidermal keratinocytes from adult donors are much more sensitive to the growth inhibitory effects of interferon than are keratinocytes from newborn donors.[41,42] This indicates that the age-associated loss of mitogenic potential may have two separate components: decreased responsiveness to mitogens and increased responsiveness to growth inhibitors.

Manifestations of photoageing in cultured skin-derived cells

Like intrinsic ageing, photoageing is reflected at the cellular level. Compared with control cells derived from sun-protected sites (upper inner arm or post-auricular area), keratinocytes or dermal fibroblasts derived from paired habitually sun-exposed sites (upper outer arm or pre-auricular area of the same donor) maintained in medium containing 10% calf serum have shorter *in-vitro* lifespans. The discrepancy increases linearly as a function of donor age and severity of clinically apparent photoageing changes.[43-45] In addition, there is a highly consistent negative effect of photoageing on dermal fibroblast growth rate and confluent density at early passage (Fig. 2), as well as an influence on cellular response to retinoic acid,[45] which is a physiological and pharmacological modulator of skin function.[46,47] The behavioural differences between previously sun-exposed and sun-protected cells of the same donor are indeed far greater and more consistent than those observed between sun-protected cells of young adult *vs* those of old adult donors, perhaps because the large, presumably genetic inter-donor variability is avoided.

Molecular manifestations of intrinsic ageing and photoageing

Intrinsic ageing and photoageing are also strongly reflected at the molecular level, at least in the model system described above. Recent studies from the authors' laboratory have examined gene expression in response to exogenous growth factors in cultured keratinocytes derived from newborn and adult sun-protected and adult sun-exposed skin. The expression of three classes of genes was determined: the proto-oncogenes *c-myc* and *c-fos*; the gene encoding the EGF receptor (*EGFr*); and the recently cloned gene *GADD 153*. Both proto-oncogenes are known to be induced in response to outside mitogenic stimuli, and are believed to be important for cell division.[48-53] The *c-fos* gene is also induced by a variety of non-mitogenic stimuli, including differentiation signals,[48,49] or ultraviolet irradiation.[50] *EGFr* is critical for

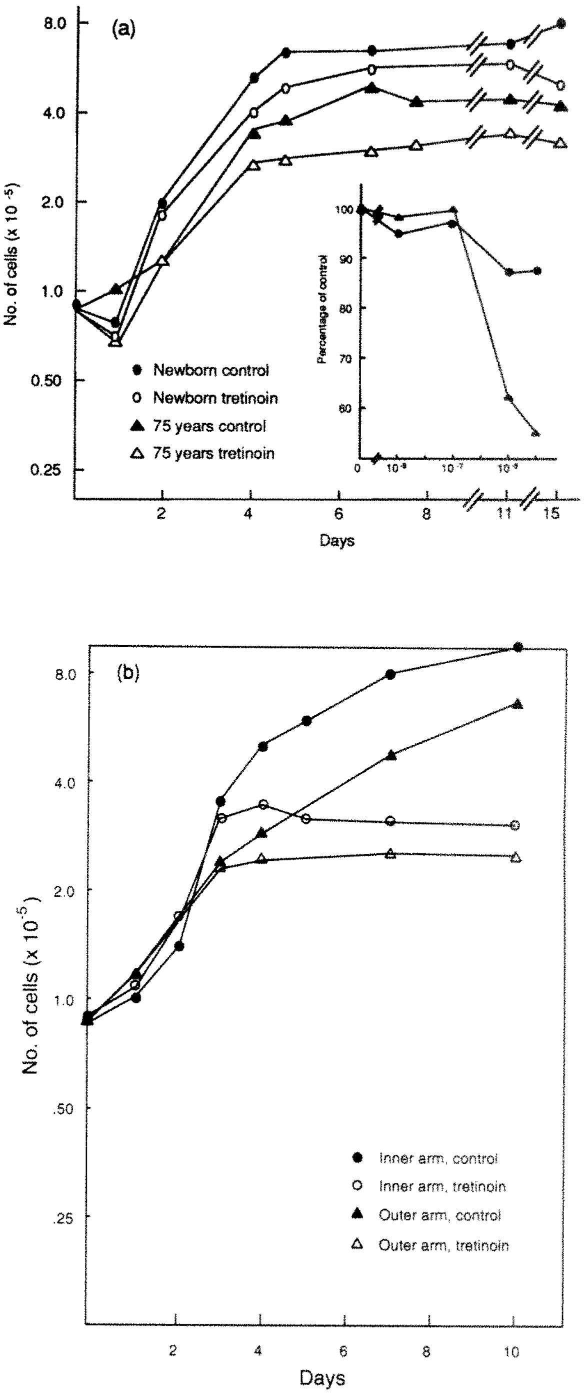

Figure 2. Influence of ageing and photoageing on dermal fibroblast growth rate, confluent density, and response to retinoic acid. (a) Fibroblasts derived from foreskin specimens of three newborns and a 75-year-old subject were maintained in medium containing 10 μM tretinoin or control vehicle alone. Inset: Dose–response for newborn *vs* old adult fibroblasts in the presence of 1 nM–100 μM tretinoin. (b) Representative growth curves of paired fibroblast cultures derived from the inner arm *vs* the outer arm of an adult donor (aged 29 years), maintained in medium in the presence or absence of 10 μM tretinoin. (Reproduced with permission from Stanulis-Praeger and Gilchrest.[45])

EGF binding and intracellular signal transduction of the mitogenic stimulus;[51] and *GADD*, so named for its induction after growth arrest and DNA damage, is known to be specifically induced in response to growth factor deprivation.[54,55]

Keratinocytes at second or third passage were grown as previously described[50] to 70–90% confluence in a serum-free medium containing 10 μg/ml EGF, 1 nM tri-iodothyronine, 10 μg/ml transferrin, 1·4 μM hydrocortisone, 10 μg/ml inositol, 10 μg/ml choline chloride and 0·09 mM calcium. Cultures were generally provided with fresh medium at 2- to 3-day intervals and 48 h prior to testing. At time 0, cultures were again provided with fresh medium and harvested for northern blot analysis immediately before and after 1, 4, 8, 14, 24 and 48 h.[48,56] Total cellular RNA (30 μg) was loaded in each lane, size separated and serially hybridized with radiolabelled complementary DNA (cDNA) probes for the messenger RNA (mRNA) species of interest. Even loading of the blots was determined by hybridization with glyceraldehyde phosphate dehydrogenase, a constitutively expressed gene.[57]

In newborn keratinocytes, *c-fos* mRNA was weakly detectable prior to stimulation, and was rapidly and strongly induced after addition of growth factors. Adult sun-protected keratinocytes also displayed induction of *c-fos* mRNA, but the level of induction was less than in newborn keratinocytes. By contrast, adult sun-exposed keratinocytes expressed *c-fos* mRNA at a relatively high level prior to mitogenic stimulation, with hardly any further induction following the addition of growth factors.

At all time points *c-myc* mRNA was easily detectable in all cultures, with a higher level of expression in newborn and young adult keratinocytes than in keratinocytes from donors aged 40 years or older. *EGFr* mRNA, also well expressed prior to stimulation, was prominently induced in newborn keratinocytes following addition of growth factors (e.g. EGF), but only slightly induced in adult keratinocytes. *GADD* mRNA was strongly expressed prior to stimulation in all cultures and was strongly down-regulated, more profoundly in newborn than in adult cells, within 2 h of stimulation. *GADD* was again maximally expressed in adult keratinocytes within 14 h after the addition of growth factors, but in newborn keratinocytes induction of *GADD* expression was only seen 24 h after growth factor stimulation, and the maximal mRNA level was attained only after 48 h.[56]

The above data suggest that, at the molecular level, adult cells respond less to outside mitogenic stimuli than do newborn cells, as expressed by reduced mRNA levels of *c-fos*, *c-myc* and *EGFr*. The reduced induction of *c-fos* in old donor cells is of particular interest in view of the fact that proliferatively senescent WI-38 cells completely failed to express this proto-oncogene after serum stimulation,[58] despite relatively intact mRNA responses for most other cell cycle-dependent genes. At the same time, adult keratinocytes perceived lack of growth factors before newborn keratinocytes, as determined by earlier maximal induction of *GADD* mRNA. Taken together, these data suggest that the marked decline in growth potential of old cells is, in part, the result of their failure to generate the steady-state mRNA levels of selected genes encoding proteins essential for progession into mitosis. Photoageing differs qualitatively from intrinsic ageing at least by its different regulation of the mRNA level for *c-fos* which is, in turn, critical for mediating a wide variety of outside signals to responsive genes.

Ageing as differentiation

If ageing is genetically programmed, as extensive data suggest it is at least in part,[59,60] then ageing at the cellular level may be considered, by definition, a form of differentiation. The term 'dysdifferentiation' has also been proposed[61] to denote 'relaxation' of gene expression, perhaps due to age-associated loss of DNA methylation. Within tissues or cultured cell populations, ageing may result from an increase in the proportion of more differentiated cells at the expense of relatively non-differentiated ones that perhaps have a greater proliferative potential and/or more functional behavioural responses, or may result from a relatively uniform shift in behaviour for all cells.

Barrandon and Green[38] have shown that with increasing donor age, individual keratinocytes, on average, have a markedly decreased proliferative potential, as manifested by generation of fewer and smaller colonies *in vitro*. Extensive studies by Bayreuther and coworkers[62,63] have defined a panel of six surface antigens for dermal fibroblasts that putatively distinguish between cells in different states of differentiation. Using immunofluorescent staining techniques, they have correlated the presence of specific markers with young *vs* old donor age, early *vs* late passage level, and high *vs* low proliferative potential. The very recent keratinocyte and fibroblast studies discussed above suggest that differentiation-like age-associated changes occur at the level of gene expression (mRNA level), as well as at the levels of proliferative response and protein expression.

Acknowledgments

This work was supported by grants from the National Institute on Aging (AG00354 and AG07114) and by the USDA Agricultural Research Service.

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British Journal of Dermatology (1992) **127**, Supplement 41, 31–36.

Long-term clinical experience with a topical retinoid

E.G.THORNE
R.W.Johnson Pharmaceutical Research Institute, Raritan, NJ, USA

Summary Topical tretinoin is a well-established treatment for acne, with a low incidence of reported adverse effects, most of which are local skin reactions. The retinoid has limited absorption through the skin, so that even with repeated applications plasma concentrations do not exceed normal endogenous levels. In mice, lifetime treatment with topical tretinoin improved skin texture and did not have any tumorigenic effects. Data from multicentre clinical trials have shown that 0·05% tretinoin emollient cream reduced fine wrinkling, surface roughness and mottled hyperpigmentation caused by photodamage. Improvement of these clinical signs was maintained after 12 months of daily tretinoin therapy, and regressed slowly after cessation of therapy. However, maintenance of the visible effects of topical tretinoin was reported after continued therapy with once or three times weekly applications of tretinoin emollient cream. Data from multicentre studies suggested that 0·1% tretinoin cream has a potential role in the treatment of solar keratoses. It is concluded that the application of tretinoin to photodamaged skin used in conjunction with sunscreens and judicious sun exposure is an effective regimen to treat the damaging cutaneous effects of chronic sun exposure.

Topical tretinoin has been used by dermatologists for over 30 years to treat a number of chronic skin diseases, including psoriasis,[1] ichthyosis,[2,3] acne[4] and others.[5] Over a period of about 22 years, more than 20 million patients have applied topical tretinoin for the treatment of acne vulgaris. Experience over this time has shown a very low incidence of serious side-effects. The most commonly reported adverse experiences have been local cutaneous reactions, such as erythema and dryness of the skin.

Patients applying tretinoin for the treatment of acne noted that their skin became softer and less wrinkled following long-term treatment. Excess exposure to ultraviolet (UV) irradiation damages the skin and results in the skin becoming rough, scaly and wrinkled with mottled hyperpigmentation. Kligman *et al.*[6] first proposed that topical tretinoin could have a potential role in the treatment of certain clinical signs associated with cumulative damage caused by years of exposure to sun, a process referred to as photodamage.

When considering the potential use of topical retinoid therapy in the treatment of photodamage, the clinician has to weigh up carefully the long-term risks against the benefits of the treatment. This paper reviews the safety profile of topical tretinoin therapy using three lines of evidence: percutaneous absorption studies; data from controlled long-term clinical studies; and animal studies. In addition, the results of studies are reviewed in which the benefits of long-term application of tretinoin were assessed in patients with mild to moderate and more severe photodamage with solar keratoses.

Safety profile

Percutaneous absorption

Clinical studies have shown that topical tretinoin is a potent drug and local reactions may occur.[7] However, the risk of systemic toxicity is low because tretinoin has limited absorption through the skin, the retinoid remaining mostly on the stratum corneum (Fig. 1).[8]

It has been shown that there is minimal systemic absorption of radiolabelled tretinoin, even following pretreatment with topical tretinoin for 28 days, or as long as 1 year.[9] Based on the measurement of radiolabel in plasma, faeces and urine, the total percutaneous absorption of tretinoin was approximately 2%. In view of the low percutaneous absorption and an average application of only 250 mg 0·05% tretinoin cream (a dose of 2·5 μg/kg for a 50-kg subject), this dose would not be sufficient to raise the normal endogenous plasma concentrations of tretinoin, which are in the range 2–5 ng/ml.[10]

Clinical experience

In published clinical studies using topical tretinoin

Correspondence: Dr E.G.Thorne, R.W.Johnson Pharmaceutical Research Institute, Route 202, P.O Box 300, Raritan, NJ 08869–0602, USA.

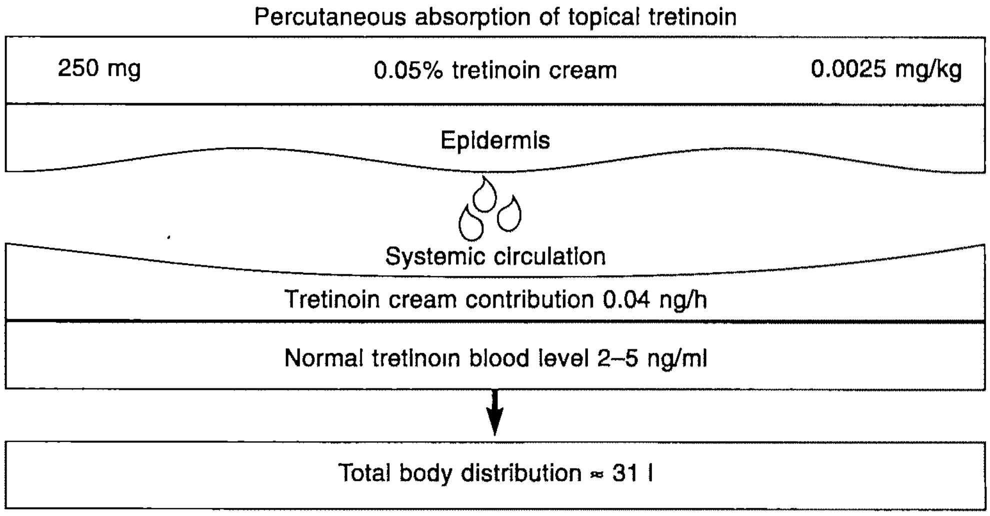
Percutaneous absorption of topical tretinoin
250 mg
0.05% tretinoin cream
0.0025 mg/kg
Epidermis
Systemic circulation
Tretinoin cream contribution 0.04 ng/h
Normal tretinoin blood level 2–5 ng/ml
Total body distribution ≈ 31 l

Figure 1. Model of tretinoin percutaneous absorption.

(Table 1), most of the 759 patients evaluated have received up to 6 months of therapy; therefore, long-term studies would be helpful to determine the risk/benefits ratio of treatment.[11–16] Recently, Ellis *et al.*[17] have reported the effects of 22 months of therapy in 16 patients. Except for the commonly reported cutaneous retinoid reaction (peeling, burning sensation and erythema), no other adverse reactions were recorded, and the patients were able to sustain the clinical improvement in visible signs of photodamage. Similar findings have been recorded in the tretinoin emollient cream studies (unpublished data) (Table 2). Of a total of 619 patients enrolled in the tretinoin emollient cream studies, 305, 212 and 82 patients remained on 0·05% tretinoin emollient cream for 6, 12 and 18 months, respectively. Mild cutaneous retinoid reactions were recorded in 50–60% of patients. The incidence peaked at 2 weeks and then gradually declined over the following 12 months. The cutaneous side-effects, although frequent, were rated as mild by both the investigators and the patients, and after treatment for 18 months only 5% of the patients had discontinued the treatment because of adverse experiences.

Table 1. Summary of investigations evaluating the effects of topical tretinoin on photodamaged skin

Author	No. of patients	Concentration of tretinoin (%)	Months of treatment
Weiss *et al.*[11]	40	0·1	4
Leyden *et al.*[12]	40	0·05	6
Lever *et al.*[13]	20	0·05	3
Weinstein *et al.*[14]	299	0·01–0·05	6
Olsen *et al.*[15]	320	0·001–0·05	6
Goldfarb *et al.*[16]	40	0·05	6

Table 2. Long-term studies involving treatment times of up to 22 months for patients applying topical tretinoin

Author	No. of patients	Concentration of tretinoin (%)	Months of treatment
Ellis *et al.*[17]	21	0.05–0.1	10
	16		22
Multicentre studies (unpublished)	305	0.05	6
	212		12
	82		18

Long-term animal studies

In addition to assessing its potential tumorigenic effect, long-term studies in mice have provided important information about the effects of applying topical tretinoin throughout most of the animal's lifetime. Such chronic animal studies should highlight any deleterious cutaneous side-effects that may not become apparent even after 20–30 years of continuous human clinical use.

In mice, two studies have been conducted to assess the lifetime application of tretinoin. The first was a tumorigenicity study performed in Japan by Tsubura and Yamamoto[18] in 1979, which involved applying 0·03 ml 0·1%

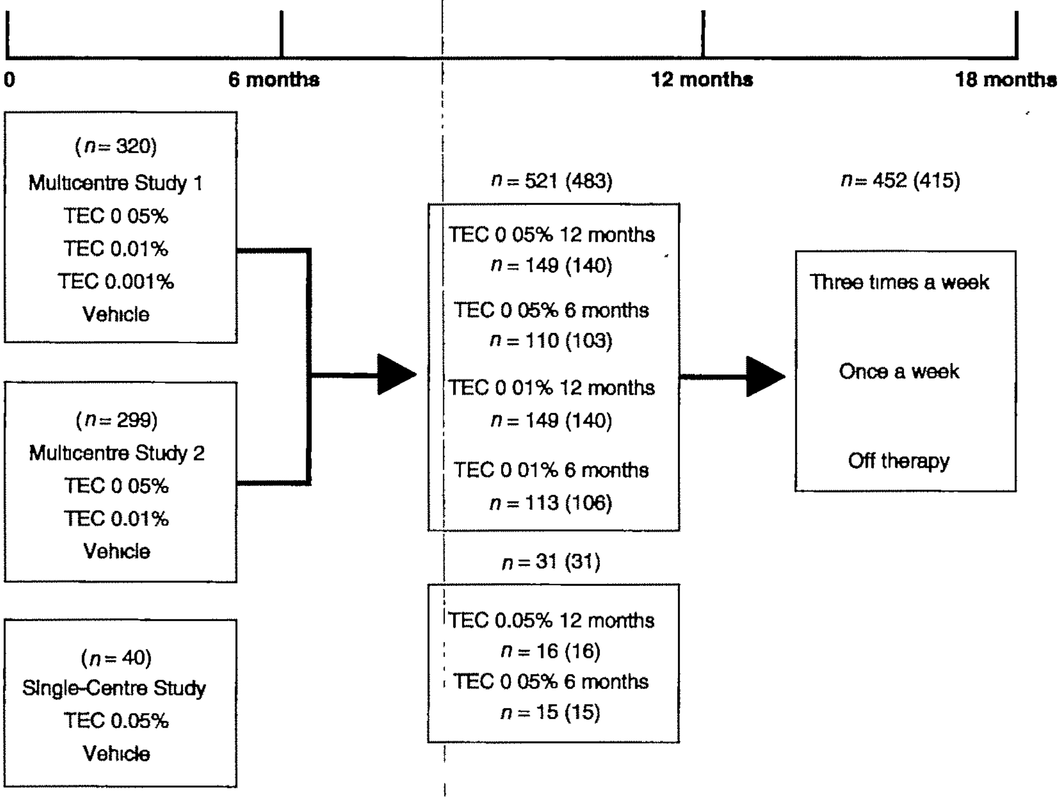

Figure 2. Summary of study design to assess the long-term effects of topical tretinoin emollient cream in the treatment of photodamaged skin. Numbers in parentheses denote the number of patients completing extensions.

tretinoin cream or lotion to ICR mice once or three times daily. Although the dose used was very high, the mice tolerated the treatment, with no evidence of tumour formation after 2 years. A second study was performed by Kligman *et al.* (unpublished data) to evaluate the effects of lifetime application of 0·025% tretinoin in hairless albino mice. In this study, 36 mice were divided into three treatment groups (tretinoin, vehicle and control) and received treatment three times weekly for 98–103 weeks. After this period, no tumours or other ill effects were noted, and longevity was unaffected. An additional finding for mice in the tretinoin treatment group was that they appeared younger and their skin was smoother. Microscopic studies confirmed the histological effects of tretinoin therapy. The tretinoin-treated skin was characterized by compaction of the stratum corneum, hypergranulosis and increased vascularization. Furthermore, the presence of a repair zone and new collagen formation were detected.

Although tretinoin in itself is not tumorigenic, it has been shown that, under certain conditions, topical tretinoin can enhance the carcinogenic effect of UV radiation in SKh-1 hairless albino mice. This mouse species is a nocturnal rodent that lacks melanin, has a thin skin and may have a compromised DNA repair mechanism. A total of 12 studies, which were of different designs, have been performed.[19] The light source, UV dose and duration of exposure, vehicle, tretinoin concentrations, treatment regimens and animal strains that were used varied, so it is difficult to draw any overall conclusion. In six of these studies there was enhanced UVB-induced tumorigenicity, whereas in three studies there was inhibition of tumour formation, and in three there was no effect.[19]

An important consideration is the control groups in these UV irradiation studies.[20–22] Mice that had not been exposed to UV radiation, but were treated with topical tretinoin for 55 weeks, did not develop cutaneous toxicity or tumours. This course of treatment, which has been calculated to be equivalent to 35 years of constant application of tretinoin in humans, did not provide evidence of any clinically significant side-effects other than the histological finding of epidermal hyperplasia, which is a typical retinoid effect.

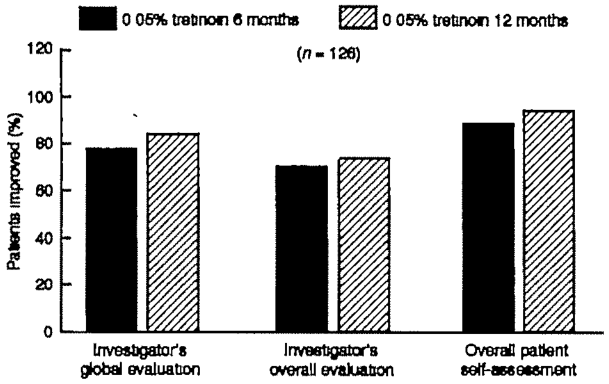

Figure 3. Key efficacy measures in a multicentre study to assess the effects of 0·05% topical tretinoin for 6 and 12 months on photodamaged skin.

Long-term clinical benefits

Photodamage

An essential approach to the prevention of photodamage is to minimize exposure to the sun, use sunscreens and wear protective clothing. Photodamage begins early in life, but the results do not become clinically apparent until the patients are aged between 30 and 50 years. At this stage fine wrinkles appear, the skin begins to feel rough, and there is irregular pigmentation. As the subject gets older, the effects of photodamage are complicated by those of chronological ageing, and premalignant and malignant changes may take place in the skin. Topical tretinoin may be effectively used to treat some of these effects of excessive exposure to the sun.

The benefits of topical tretinoin emollient cream for the treatment of mild and moderate photodamage have been studied in two multicentre studies and one single-centre study.[14–16] In addition to the clinical parameters, silicone skin surface replicas provided further evidence for tretinoin's effects on the surface of photodamaged facial skin.[23] Findings from baseline biopsies and those at the end of 6 months of tretinoin therapy helped to characterize the alterations in skin structure.[24] These reports detailed the clinical outcome using 0·001% ($n = 80$), 0·01% ($n = 180$) and 0·05% ($n = 200$) tretinoin compared with vehicle treatment ($n = 199$) over a period of 6 months. In an effort to obtain long-term safety data, the initial study period of 6 months was extended for an additional 6 months and randomly assigned patients ($n = 223$) received either 0·05% or 0·01% tretinoin treatment. Patients ($n = 149$) originally assigned to 0·01% and 0·05% tretinoin continued treatment for a total of 1 year.

To determine if the improvement in appearance of photodamaged skin noted by the investigators and the patients receiving topical tretinoin emollient cream regressed after cessation of therapy, patients ($n = 452$) were randomly assigned either to stop tretinoin therapy or to apply 0·05% or 0·01% tretinoin once or three times weekly (Fig. 2).

Throughout the study, the key efficacy parameters were the investigator's global evaluation, patients' self-assessment at week 24, and the investigator's rating of overall severity of photodamage at each visit. Based on data from vehicle-controlled studies, 0·05% tretinoin provided the most consistent improvement after 6 months of therapy. About 79% of patients showed global improvement, 81% of patients assessed themselves as improved, and the overall severity improved in 69% of patients applying 0·05% tretinoin emollient cream for 6 months. For the 126 patients who completed 12 months of therapy with 0·05% tretinoin cream, only a small increase in the number of patients showing improvement was noted. However, the benefits seen during the first 6 months of therapy persisted, suggesting the maintenance of the retinoid effect (Fig. 3).

If tretinoin emollient cream applications were discontinued after 12 months, few patients or investigators considered that there was further improvement. Importantly, the clinical benefits were maintained even 6 months after the termination of therapy (Fig. 4). At this time, the investigators considered that 47% of patients maintained the improvement in visible signs of ageing (fine wrinkling, roughness and mottled hyperpigmentation), compared with 35% of patients who felt that they had maintained their improvement despite discontinuing therapy.

In patients applying 0·05% tretinoin emollient cream once weekly, investigators rated 68% of patients as having maintained the benefits or improved during the 6 months of altered therapy. About the same improvement or maintenance rate was noted by the patients. For those patients in the three-times-weekly treatment group, a higher percentage of patients than investigators thought that there had been maintenance or improvement (90% of self-assessed patients *vs* 77% of investigator-rated patients).

Solar keratoses

In addition to photodamage, excess exposure to the sun can result in solar keratoses, characterized by prominent red or flesh-coloured, warty lesions. These premalignant lesions show histological variation in cell size, nuclear

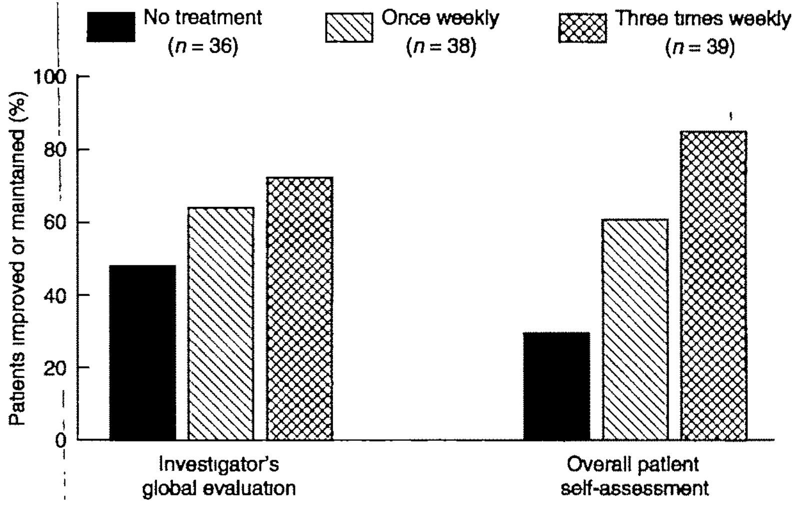

Figure 4. (a) Investigators' global evaluation and (b) patients' self-assessment of photodamaged skin 6 months after withdrawing treatment or altering treatment from 0·05% tretinoin once daily to application once or three times weekly.

heterogeneity and loss of polarity. It is therefore considered that a small proportion may eventuate into squamous cell carcinoma.[25,26]

A number of trials have been undertaken to assess the effects of tretinoin in the treatment of patients with solar keratosis.[27] Over the last 6 years, a total of 1265 patients have been evaluated in double-blind studies at 31 different centres and treated with 0·05% or 0·1% cream for periods of up to 15 months. In all cases, the diagnosis of solar keratosis was confirmed by histological examination at the start of the trial. The efficacy parameters used to evaluate the treatment were lesion count, investigators' global evaluation and patients' self-assessment. Analysis of the data showed that treatment with 0·1% tretinoin cream applied twice daily for 6 months was the most effective treatment, resulting in a significant ($P<0{\cdot}001$) reduction in solar keratoses compared with vehicle; reductions in lesions in the tretinoin and vehicle treatment groups were 56% and 41%, respectively. Clinical evaluation also showed that tretinoin was significantly ($P<0{\cdot}001$) superior to vehicle, with an excellent or good response being reported in 73% and 40% of cases, respectively. It is noteworthy that there was a relatively high response rate in the control, vehicle-treated group, as has been observed in other studies.[27] A possible explanation for this is that the patients were instructed to apply sunscreens and to avoid exposure to the sun.

As in the studies performed to assess the use of tretinoin for the treatment of photodamage, the cutaneous side-effects of tretinoin were well tolerated when used for the treatment of solar keratoses. Only eight of the 455 patients had to withdraw from treatment, although 88 patients reduced the application frequency of 0·1% tretinoin cream because of local irritation.

Conclusions

The fact that topical tretinoin has, over 30 years of clinical use, shown no evidence of any chronic toxicity is supported by long-term toxicological studies in mice, which have shown that topically applied tretinoin was not carcinogenic. In some studies, however, tretinoin enhanced the tumorigenic effect of UVB irradiation. Topically applied tretinoin has been successful in treatment of some of the visible signs of photodamaged skin and solar keratoses, but the concomitant use of a sunscreen and the avoidance of the sun should always be recommended, and the general awareness of a healthy skin should be promoted by the dermatologist. Although some patients experienced local cutaneous reactions initially, it can be concluded that the long-term benefits of tretinoin outweigh any risks.

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The clinical identification and quantification of photodamage

C.E.M.GRIFFITHS
Dermatopharmacology Unit, Department of Dermatology, University of Michigan Medical Center, Ann Arbor, Michigan, USA

Summary The distinction between intrinsic and extrinsic ageing can be made on both histological and clinical grounds. Clinical criteria associated with the diagnosis of extrinsic ageing are coarse wrinkles, actinic lentigines, elastotic conditions, purpura, telangiectasia and cutaneous neoplasms. These parameters are always superimposed on changes associated with intrinsic ageing: namely, fine wrinkles and benign growths. There is heightened interest in extrinsic ageing as a result of studies demonstrating the efficacy of topical tretinoin in improving this condition. As a consequence, systems for grading extrinsic ageing have been developed, including a photographic standard scale which removes some of the subjectivity inherent to current methodology.

Cutaneous ageing is a process that is a seemingly inevitable consequence of senescence. However, this 'natural' event can be divided into two components: physiological and pathological. Intrinsic or chronological ageing, which is related purely to senescence, genetics accounting for the age of onset and susceptibility, occurs in its pure form in non-sun-exposed body areas, such as the buttocks. Extrinsic ageing, also known as photodamage, is directly related to environmental factors and occurs in habitually exposed areas of the body, such as the face, neck and arms. Extrinsic ageing will indubitably always be superimposed on a background of intrinsic ageing.

The first descriptions of extrinsic ageing or photodamage became available from the end of the 19th century, when it was referred to as farmer's or sailor's skin.[1] At that time, photodamage was only readily observed on people with outdoor occupations and, by and large, was not seen in the middle or upper social classes, except for golfers, cricketers and other sportsmen. Women from the non-working classes were rarely photodamaged because sensible sun avoidance was practised, as evidenced by the wearing of long skirts, long sleeves and hats, and the use of parasols. These features may have been the dictates of fashion and prudery, but were also very satisfactory sun-avoidance measures. Towards the middle of the 20th century, with the introduction of more leisure time and a life style centred around seaside vacations and outdoor pursuits, there was an inevitable increase in sun exposure. A sun-tan became a desirable goal redolent of a 'healthy', monied existence; indeed, this aura of a sun-tan indicating a 'jet-set' life style still persists. As a consequence, the effects of prolonged sun exposure became associated with the development of wrinkles and dyspigmentation—a process misinterpreted as accelerated ageing. The misconception persists today and is often referred to as 'premature ageing'.

This article delineates the clinical differences between intrinsic and extrinsic ageing, and discusses the technology currently available to help quantify extrinsic ageing.

Definition of cutaneous ageing

Intrinsic or chronological ageing is observed on those areas of the skin that are habitually covered and not exposed to sun or other environmental factors. In these areas, ageing is characteristically manifested by smooth, pale and finely wrinkled skin, superimposed on which may be benign growths, such as cherry haemangiomas and seborrhoeic keratoses. By contrast, extrinsically aged or photodamaged skin is remarkable for its coarseness, the principle clinical features being roughness, sallowness, fine and coarse wrinkles, hyper- and hypopigmentation, purpura, telangiectasia and a variety of benign and malignant neoplasms (Table 1).[2]

The histological changes inherent to intrinsic and extrinsic ageing are dealt with in more detail elsewhere in this supplement in the article by Professor Marks.[3] As a general rule, in intrinsic ageing there is a reduction in all histological parameters—to quote Kligman,[4] 'in intrinsic ageing everything shrinks'—whereas in extrin-

Correspondence: Dr C.E.M.Griffiths, Department of Dermatology, University of Michigan Medical Center, 1910 Taubman Center, 1500 East Medical Center Drive, Ann Arbor, Michigan 48109–0314, USA.

Table 1. Clinical differentiation of extrinsically and intrinsically aged skin

Extrinsically aged	Intrinsically aged
Wrinkles—coarse and fine	Wrinkles—fine
Rough and dry	Smooth and soft
Dark, leathery	Pale
Dyspigmentation	
Telangiectasia	
Actinic keratoses	

sic ageing there is an increase in histological parameters. The salient histological features of extrinsically aged skin are an initial thickening of the epidermis, but at the end-stages the epidermis becomes atrophic and is accompanied by keratinocyte atypia, actinic keratoses and neoplasms, such as basal and squamous cell carcinomas. Melanocytes increase in number, whereas there is a reduction in Langerhans cells by as much as 50% in photodamaged as compared with intrinsically aged skin.[5] The most striking changes occur in the dermis. In intrinsically aged skin, there is a marked reduction and homogenization of collagen within the papillary dermis. In extrinsically aged skin, however, there is an initial excess deposition of microfibrillar elastic fibres proceeding to a disorganized mass of truncated, functionless elastic material. These profound changes in elastic tissue are set amidst an increased deposition of proteoglycans and glycosaminoglycans.[6] In foetal skin these substances are greatly increased, gradually diminishing with age, whereas in photodamaged skin there is an increase almost to prenatal values. The other salient distinguishing feature of photodamaged skin is that of heliodermatitis.[7] Within the dermis of early and moderately photodamaged skin such as 'red neck', there is a peri-appendageal and perivascular infiltrate composed of neutrophils, lymphocytes and degranulating mast cells. This infiltrate is not seen in chronological ageing. It is postulated that cytokines and enzymes released from these infiltrating cells are, at least in part, responsible for the degradation of elastic tissue.

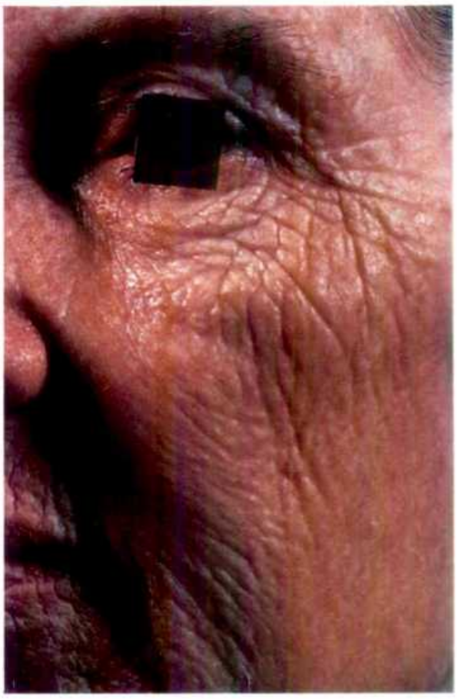

Figure 2. Coarse wrinkling—the characteristic feature of extrinsic ageing.

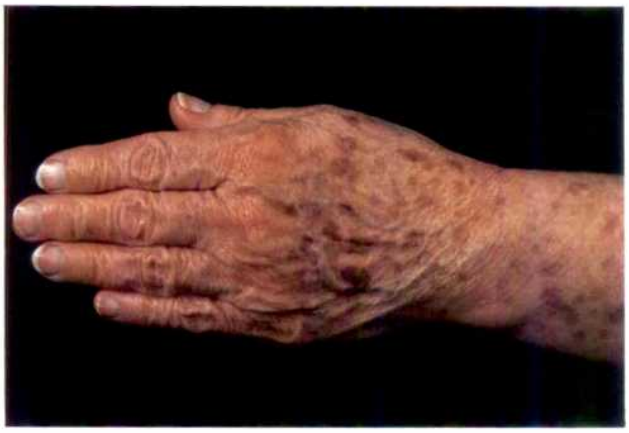

Figure 1. Actinic dyspigmentation consisting of actinic lentigines and flat, pigmented seborrhoeic keratoses.

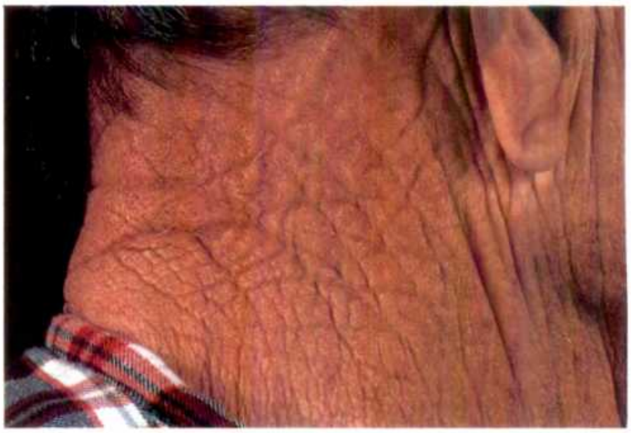

Figure 3. Cutis rhomboidalis nuchae in an outdoor worker.

Features of extrinsic ageing

Hyperpigmented lesions

Chronically sun-exposed skin is characteristically speckled with freckles, lentigines and flat seborrhoeic keratoses set in a background of diffuse mottling (Fig. 1). These pigmented lesions can be clinically very difficult to distinguish from one another. In a recent study of the efficacy of topical tretinoin in the treatment of actinic lentigines, despite careful clinical selection of what were perceived as lentigines, only 60% of these lesions fulfilled histological criteria for the diagnosis, the rest being classified as seborrhoeic keratoses, or unclassifiable.[8] Actinic lentigines are erroneously referred to as 'age spots' or 'liver spots', but are not so much a manifestation of age as of photodamage—they are not seen in sun-protected areas. Although seborrhoeic keratoses are frequently observed on photodamaged skin, they are also found in sun-protected areas and, as such, are a concomitant but not a diagnostic feature of extrinsic ageing.

Wrinkles

Coarse wrinkles are usually found on the forehead and in the periorbital and perioral areas, the so-called animation patterns, and are more indicative of extrinsic damage than are fine wrinkles.[9] The postulation for coarse wrinkles following these patterns is that the furrow of each wrinkle is relatively spared from ultraviolet exposure, in that solar elastotic material is greatest at the edge of wrinkles and least in the wrinkle floor.[10] Coarse wrinkles can be distinguished from fine wrinkles by the inability to efface them by stretching between two fingers; fine wrinkles are completely effaced using this procedure. Coarse wrinkles must also be differentiated from the sags and jowls associated with gravitational pull—a feature of intrinsic ageing. Fine wrinkles, as well as being associated with intrinsic ageing, are a particular feature of photodamage in women, particularly a reticulate pattern on the cheeks. Coarse wrinkling *per se* is probably the pathognomonic feature of extrinsic ageing (Fig. 2), although it is not unique to sun damage.

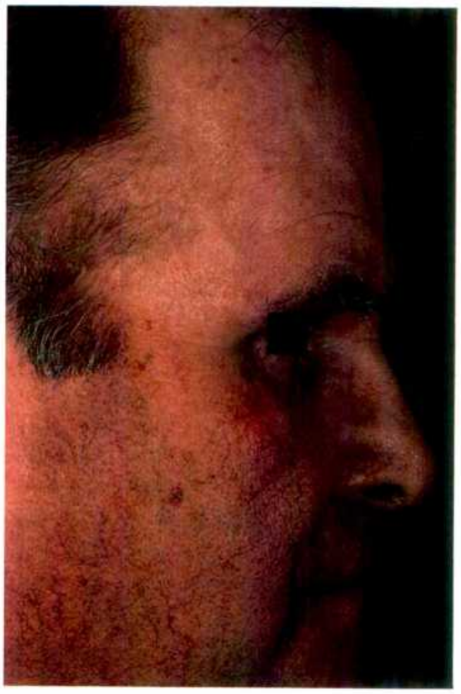

Figure 5. Telangiectasia of the cheeks—an associated but not a pathognomonic feature of extrinsic ageing.

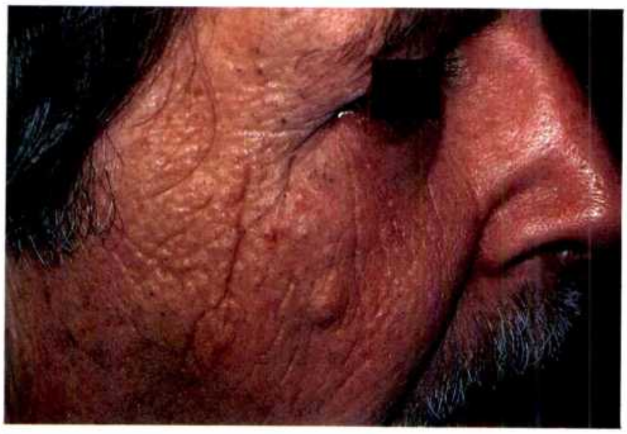

Figure 4. Favre–Racouchot syndrome: note the comedones and epidermoid cyst set among the coarse wrinkles of extrinsic ageing.

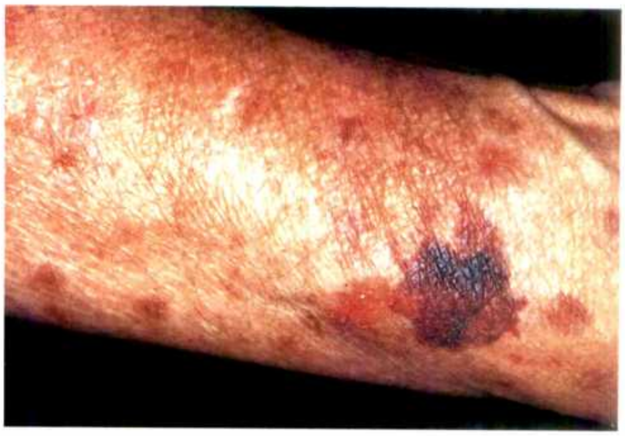

Figure 6. Bateman's purpura and concomitant stellate pseudoscars.

Kligman[11] has demonstrated in guinea pigs that heat in the form of infra-red radiation can produce similar increases in dermal elastic tissue to those observed with ultraviolet irradiation. Attention has recently been focused on the association between smoking and coarse facial wrinkling. The pathogenesis of this association is unknown at present, but is most probably multifactorial, relating to changes in dermal capillaries, direct oxidant effects of cigarette smoke, local heat and mechanical factors, such as squinting.[12]

Elastotic conditions

As previously mentioned, the major histological change seen in photodamaged skin is one of increased, non-functional elastotic tissue which gives rise to a variety of clinical conditions in addition to a reduction in cutaneous elasticity. The back of the neck, with its continuous exposure to sun, particularly in the case of men working outdoors and golfers, initially manifests photodamage as 'red neck', which in its latter stages takes on a characteristic appearance of deep geometric furrows set among yellowed elastotic skin—a condition known as cutis rhomboidalis nuchae (Fig. 3).

The lateral periorbital region may develop a unique elastotic condition of its own, with the appearance of comedones and epidermoid cysts set in a deeply wrinkled sallow complexion. This constellation of features is known as the Favre–Racouchot syndrome (Fig. 4), which most probably arises as a result of ectatic sebaceous follicles developing into cysts or filling with keratinaceous debris and forming comedones.

Solar elastosis in its commonest form occurs in fair-skinned individuals and is observed as a pale yellow, copper-beaten appearance of the skin particularly overlying the temples—a result of increased visibility of yellow elastotic debris viewed through an atrophic epidermis and relatively avascular papillary dermis.

Sebaceous hyperplasia

These distinctive, yellowish, umbilicated papules, 2–3 mm in diameter, often with a central punctum, occur most commonly on the forehead and cheeks. They result from enlargement of a single sebaceous gland comprising multiple lobules around a single enlarged duct.[13]

Vascular changes

In both intrinsic and extrinsic ageing, the superficial horizontal capillary plexus is markedly reduced. In intrinsic ageing this is all that occurs and accounts for the pallor of intrinsically aged skin and, most probably, the sallowness of extrinsic ageing. In extrinsic ageing, the vessels become tortuous and dilated, producing telangiectasia, which is most commonly observed on the nose and cheeks (Fig. 5), but is not solely a result of environmental factors, since liver failure, high oestrogen concentrations and scleroderma, among other conditions, may produce the same appearance. On the pinna and lower lip, the vessels may break down further to produce venous lakes. In severe extrinsic damage, the vessel wall thickens as a result of reduplication of the basement membrane.[4] This increased vascular rigidity coupled with a reduction in supportive, perivascular connective tissue results in fragility of the dermal vessels and, consequently, purpura following even the slightest injury. The purpura associated with photodamage is referred to as Bateman's purpura (Fig. 6).

Pseudoscars

These stellate or angulated lesions, typically found on photodamaged forearms, are true scars,[14] developing from trauma and subsequent haemorrhage, the result of a reduction in both vascular supply and connective tissue due to poor wound healing. As a consequence, pseudoscars are frequently observed adjacent to Bateman's purpura (Fig. 6).

Actinic keratoses

Also known as solar keratoses, these are a common premalignant condition. There is a strong relationship between exposure to ultraviolet radiation and actinic keratoses, although they may be associated with ingestion of arsenic and local heat to the skin. The correlation with skin type is high and is particularly prevalent among skin types I–II. A study by Marks and colleagues[15] in Australia found that actinic keratoses occurred in approximately 60% of white Australians aged 40 years or over. Actinic keratoses appear as small, pink, scaling papules, occasionally coalescing into plaques, on sun-exposed areas of the face and extremities. The potential to transform into a squamous cell carcinoma is small, yet real, but it should be borne in mind that the 'normal', adjacent, sun-damaged skin is probably just as likely to produce a squamous cell carcinoma.

Basal cell and squamous cell carcinomas

A detailed discussion of these cutaneous malignancies is beyond the scope of this article, but it should be stated that both these tumours are strongly correlated with chronic sun exposure and, as such, are more commonly, although not exclusively, found in areas of extrinsic ageing.

Assessment of photodamage

The increasing public and medical awareness of cutaneous photodamage is partly a result of educational campaigns relating to skin cancer and the proven use of agents, such as topical tretinoin, to reverse some of the features of extrinsic ageing.[16,17] Heightened awareness of this and resultant dermatopharmacological research into photodamage has made it necessary to clarify and quantify these changes in the form of grading scales.

The assessment of extrinsic ageing is not a clinically taught skill and, until recently, has relied on the use of descriptive scales such as that developed by the R.W. Johnson Pharmaceutical Research Institute, which grades the various parameters of extrinsic ageing to produce an overall assessment on a 10-point scale, where 0 denotes none, 1–3 denotes mild, 4–6 denotes moderate and 7–9 denotes severe.[18] Although a satisfactory scale, this technique relies on the experience of the investigator and is somewhat subjective. Prior experience with photographic standard scales for the assessment of acne[18] demonstrated the usefulness of such visual scales for the grading of cutaneous disease. Such a scale had previously been described for the grading of wrinkles or the 'crow's feet' of the lateral periorbital region of the face. This scale, developed by Daniell,[19] used a 1–6 grading scale for the quantitation of crow's feet wrinkles associated with smoking, and demonstrated good reproducibility. In the author's photoageing clinic, a photonumerical scale has been developed which is based on five photographic standards illustrating grades of facial photodamage from 0 (none) to 8 (severe). In a side-by-side comparison with the existing descriptive scale, the photonumerical scale was found to have superior inter-grader agreement. However, the repeatability between the two scales was not significantly different 1 week later.[20] This scale is useful for categorizing subjects into different grades of photodamage, but can only be used to assess major improvements resulting from the use of skin repair agents. As a clinical measure, this is currently the best available, but is in itself a non-linear grading, and only semi-quantitative.

Quantitative methods available for determining the severity of photodamage are reliant on image analysis and subsequent digitization; thus, the results are not often instantly accessible. One such method is optical profilometry, developed by Grove *et al.*,[21] and now used extensively in studies of photoageing treatment. This method measures both fine and coarse wrinkles by taking a silicone rubber cast of a 'target area' of the face and subjecting the resulting impression to computerized digital image processing. The wrinkles are read as 'north–south' or 'east–west' shadowing under oblique light, with the north–south facet showing the best correlation with a clinical grading based on the Daniell system.[21] A drawback of this system is that no objective units are given and, as such, it is difficult to interpret in isolation.

Other methods for quantitation of, skin topography also rely on the use of skin surface replicas and subsequent computer-assisted analysis. The method described by Murphy *et al.*,[22] which is reliant on these techniques, would most probably be of use in the assessment of skin texture, particularly the smoothness induced by topical tretinoin, one of the earliest and most consistent features of induced improvement. The development of high-resolution television pictures of the skin would allow subsequent digitization of wrinkles and quantification, although this technique is still dependent on the ability to assess the same area from visit to visit, i.e. its reproducibility.

Although wrinkles are difficult to quantify, other features of photodamage—namely, actinic lentigines—lend themselves more readily to quantification. In addition to straightforward counting of the individual lesions, there are measures of colour. Colorimeters can be used as an objective measure of colour reflectance or intensity. One particular form of colorimeter (Chroma-Meter, CR, 200b, Minolta Camera Co., Osaka, Japan) is in constant use in the author's photoageing clinic and has proved to be reliable in the documentation of pigment changes in actinic lentigines, melasma and post-inflammatory hyperpigmentation during studies on the use of topical tretinoin for these indications (S.M.Bulengo-Ransby, unpublished data). Epidermal pigment can be distinguished from dermal pigment by the use of a Wood's light: epidermal pigment is enhanced under this form of lighting, whereas dermal pigment shows no discernible change. A Wood's light also helps to visualize subclinical lesions such as ephelides or lentigines that are not readily discernible under normal lighting conditions. Indeed, when patients view their skin under Wood's light, they are frequently astounded at the degree of photodamage not previously evident.

As research progresses, the molecular basis for intrinsic and extrinsic ageing will be elucidated, thereby helping to delineate even more accurately the differences between these two conditions. The ability to quantify the clinical changes inherent to extrinsic ageing are still rudimentary, but the application of sophisticated computer analysis to this problem will surely produce an objective, quantitative approach to classification in the near future.

Acknowledgment

This study was supported in part by grants from the R.W.Johnson Pharmaceutical Research Institute, Raritan, N.J., and the Babcock Dermatologic Endowment, Ann Arbor, Michigan.

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British Journal of Dermatology (1992) 127, Supplement 41, 43–47.

Retinoic acid and pigment cells: a review of *in-vitro* and *in-vivo* studies

J.-P.ORTONNE
Service de Dermatologie, Hôpital Pasteur, Nice, France

Summary Topical tretinoin improves mottling and hyperpigmented lesions of photodamaged skin. The basic mechanisms underlying these effects are not known. It is demonstrated that retinoids inhibit the growth and enhance the differentiation of melanoma cells *in vitro*, and stimulate the constitutive melanogenesis in melanoma cells *in vitro*. On the other hand, they inhibit hormonally or pharmacologically induced melanogenesis in these cells. Very few data are available concerning the effect of retinoic acid on normal human melanocytes, but there is some inhibition of growth as in melanoma cells. Retinoic acid appears to have little effect on the melanogenesis of normal human melanocytes grown *in vitro* using serum-free culture medium. Changes in the shape of these melanocytes suggest that retinoic acid acts on cytoskeleton proteins. Further studies, both *in vitro* and *in vivo*, are needed to clarify the effects of retinoic acid on melanocytes.

Pigmentary changes are one of the visible markers of photoaged skin.[1–3] Photodamaged skin is typically heterogeneous in colour, with a mottled appearance,[2,3] and actinic lentigines are the most common reason for patients seeking treatment.

Several clinical studies have demonstrated that topical tretinoin improves the irregular hyperpigmentation associated with photoageing,[4,5] the effect being dose-dependent.[6] More recently, a significant lightening of hyperpigmented lesions associated with photodamage has been reported in patients treated for 10 months with a once-daily application of 0·1% all-*trans*-retinoic acid (RA) cream,[7] and no recurrences were detected during a 6-month follow-up period after the discontinuation of therapy. The mechanism of action of tretinoin on the pigmentary abnormalities of photoaged skin has yet to be elucidated.

Various hypotheses have been proposed, such as a decrease in melanosome transfer to keratinocytes secondary to an increased epidermal turnover. This is further suggested by the effect of topical tretinoin on pigment macules resembling human freckles that occur on faces of macaques.[8] The hyperpigmentation is due to an epidermal hypermelanosis, with an increased number of well-developed DOPA-positive melanocytes. Interestingly, topical 0·1% tretinoin has been shown to induce significant depigmentation of these lesions as early as 1 month after commencing treatment. Morphological studies have not been able to demonstrate any changes in the size, number and melanogenic activity of the melanocytes, despite a marked reduction in epidermal melanin pigmentation. As the number of S-phase basal cells has been shown to increase by approximately 50% after tretinoin treatment, it has been suggested that RA-induced lightening of the macules may be largely due to the dispersion of melanin granules in the acanthotic keratinocytes and their fast turnover.[8] However, this is probably too simplistic a view, and it is not yet established whether these effects of RA on photoaged skin result from its direct effect on melanocytes, or from an indirect action via keratinocytes and/or keratinocyte-derived factors, or via dermal influences.

Another noteworthy observation is that topical 0·1% tretinoin appears to stimulate melanogenesis in Yucatan pigs.[9] Electron microscopy has revealed an increase in the number and size of melanocytes in tretinoin-treated areas of skin. The melanosomes are also larger and more abundant within the melanocytes and keratinocytes. The reason for this disparity between the response in human and pig skin is not yet known.

Clinical trials have shown that topical tretinoin can modify dysplastic or malignant melanocytes. Fading of some lesions, or the elimination of some dysplastic nevi with histological evidence of the disappearance, or reversion to benign nevi has been reported following tretinoin treatment.[10] The daily application of a 0·05% tretinoin solution to cutaneous melanoma metastases for 4 months induced significant regression in two patients.[11] These observations, which are of no practical

Correspondence: Professor J.P.Ortonne, Service de Dermatologie, Hôpital Pasteur, B.P.No. 69, 06002 Nice Cedex 1, France.

value for the treatment of these pigmented lesions, suggest a definite biological effect of topical tretinoin on pigment cells.[10]

Only a few studies on the action of RA on the proliferation and melanogenic activity of pigment cells have been reported. The studies have generally been performed using melanoma cells, and it is difficult to extrapolate the results obtained to normal human melanocytes. Despite these limitations, these studies may help to improve our understanding of the effects of RA on the melanin pigmentary system in man.

Melanocytes in photoaged skin: morphological study

Light microscopy studies of photodamaged skin have demonstrated melanocyte hypertrophy and hyperplasia associated with a heterogeneous distribution of melanin granules.[4] The distribution of melanocytes along the basal layer is irregular, and this is probably the reason for the characteristic mottled appearance of aged skin that has been chronically exposed to the sun. Using electron microscopy, it has been shown that there is considerable heterogeneity of the morphological and functional characteristics of melanocytes.[12] Most of these cells are very large and undergo changes indicative of hyperstimulation, whereas others show little evidence of melanogenesis; the cells contain only a few melanized melanosomes, and have no dendrites.

After RA treatment, the size and number of epidermal melanocytes decrease.[4] In a 24-week study during which three different tretinoin concentrations were applied, a significant dose-dependent decrease in the epidermal melanin content was observed.[6] This modification appeared to be stable, the decline in epidermal melanin content still being present after 48 weeks, whereas other histological changes, such as stratum corneum compaction and increases in the thickness of the epidermis and granular layer, returned to baseline after completion of treatment.[13] Only treatment with 0·001% tretinoin produced no effect on melanin pigmentation. This observation suggests that there is a decrease in the melanogenic activity of melanocytes as a result of RA treatment. The findings that, in photoaged skin after topical tretinoin treatment, melanocytes had fewer dendritic processes and fewer and more immature melanosomes, also support this hypothesis.[14]

Topical 0·1% tretinoin can significantly improve the microscopic features of hyperpigmented lesions associated with photodamage. These include actinic lentigines and non-lentiginous lesions. A statistically significant reduction in epidermal melanin pigmentation has been observed in these two types of lesion.[7] However, no changes in the number and size of melanocytes have been detected. Furthermore, the degree of clubbing of rete ridges, a modification associated with actinic lentigines, remained unchanged after tretinoin treatment.

Retinoic acid and melanogenesis in pigment cells

Several biochemical studies have been performed on different melanoma cell lines *in vitro* to evaluate the effect of retinoids on melanin synthesis. Retinoic acid has been reported to either stimulate or suppress melanization in these cells.[15–18]

In response to RA (10^{-6} M for 5 days), the murine melanoma cell lines B16-F1 and S91-C2 exhibited increased tyrosinase activity and melanin synthesis.[15,16] In contrast, there was no melanogenic effect on K-1735P ultraviolet-irradiated, transformed murine melanoma cells, which remained amelanotic following RA treatment.[19]

Another study has demonstrated that, in the melanoma cell lines SK Mel-28 and A375, 14-day treatment with 10^{-7} and 10^{-6} M RA and acitretin significantly enhanced melanin synthesis. In this system, the other retinoids—isotretinoin, temarotene, Ro 13–7410 and Ro 14–6113—had no significant effects on melanin production.[20] It has been established, however, that melanoma cells showed a different susceptibility to RA-induced changes in melanotic expression.[16,17] An inhibition of melanogenesis has also been demonstrated in some melanoma cell lines. The effect of RA on melanization induced either hormonally or pharmacologically has also been studied.[21] In a study using Cloudman S91 melanoma cells, it was demonstrated that RA inhibited the induction of melanogenesis in response to melanocyte-stimulating hormone, cholera toxin and isobutylmethylxanthine. More specifically, RA inhibited the activation of tyrosinase and DOPA chrome isomerase; this was not the result of a direct inhibition of these enzymes or the uptake of tyrosine. Despite this inhibitory effect, RA stimulates the binding of melanocyte-stimulating hormone to S91 melanoma cells.[22,23] At the ultrastructural level, the maturation of melanosomes is blocked at stage II, and no tyrosinase activity has been detected in trans Golgi and coated vesicles of stimulated melanoma cells.

The main conclusion to be drawn from these studies of melanoma cells, therefore, is that RA probably affects the melanogenesis via a direct effect on pigment cells. Due to the specific reactivity of each cell line, however, it is difficult to establish an unequivocal effect of RA on the melanogenic activity in these cells.

Few data are at present available on the effects of RA on normal human melanocytes. In a review by Yaar and Gilchrest,[24] it is stated that retinoids have not been demonstrated to have any effect in these cells. When treated with RA, epidermal melanocytes grown in a serum-free culture medium which was supplemented with foetal growth factor, bovine pituitary extract, insulin and phorbol myristate acetate did not display significant changes in either the melanin content or tyrosinase activity. This suggests that RA does not play a major role in regulating constitutive melanogenesis of normal human melanocytes.[25]

To date, the presumptive second-messenger pathway for melanogenesis has not been clearly identified. There is no evidence that human melanogenesis is mediated principally via the cyclic adenosine monophosphate (cAMP) pathway. However, there are substantial data to indicate that at least some melanogenic signals are transmitted instead via the 1,2-diacylglycerol–protein kinase C pathway. Recent data suggest that the β-isoform of protein kinase C may specifically mediate melanogenesis,[26] and levels of the α-form messenger RNA in these cells are simultaneously decreased with the reduction in melanin polymer formation.[27] It has also been suggested on the basis of these results that the increased activity of protein kinase C induced by RA plays a critical role in the inhibitory effect on melanogenesis in melanocytes.

It is interesting to note that there is evidence that topical tretinoin treatment affects these cells' transduction pathways in human skin *in vivo*.[28] From the study of topical retinoic acid-treated human skin, it has been demonstrated that phosphoinositide-specific phospholipase C activity and the 1,2-diacylglycerol content of melanocytes are elevated, and that protein kinase C activity is reduced.[29] Based on these observations, therefore, an *in-vivo* effect of topical retinoic acid on melanogenesis would be expected.

Retinoic acids, retinoids and proliferation of pigment cells

In B16 mouse melanoma cells, RA inhibits proliferation specifically in the G1 phase of the cell cycle.[29] Another murine melanoma cell line, S91-C2, is highly sensitive to RA, with growth being suppressed by RA.[30] A similar observation has also been made in the case of K-1735P cells.[19]

In the human melanoma cell lines SK Mel-28 and A-375, exposure to 10^{-7}–10^{-4} M tretinoin for 4 days resulted in a complete lack of any antiproliferative activity, and acitretin exposure produced only a low-grade inhibition of growth in one of these two cell lines. By contrast, other retinoids (isotretinoin, temarotene, Ro 13-7410, Ro 13-6307 and Ro 14-6113) inhibited growth by 50% in this system.[20] Proliferation of normal human melanocytes grown in serum-free culture medium was also inhibited by RA.[25]

The mechanism whereby RA induces growth inhibition in B16 melanoma cells *in vitro* has been further examined. In these cells RA induces protein kinase C,[31,32] and also increases the amount of cAMP-dependent protein kinase.[33] The recent finding that B16 mouse melanoma cells, selected for resistance to the growth inhibition mediated by cAMP, are cross-resistant to RA-induced growth inhibition suggests that cAMP regulates some step in the RA signal transduction pathway in these cells.[34]

Retinoic acid and migration of pigment cells

The role of tumour cell locomotion in invasion and metastasis has been widely studied in various tumour cells, including malignant pigment cells. It has been demonstrated that RA inhibits the migration of B16 murine melanoma cells *in vitro* when assayed on polycarbonate membranes.[35] In another assay system on type I collagen, all-*trans*-RA inhibited the growth and infiltration into the gel of murine melanoma cells (B16 and Cloudman S91),[36] and a similar finding has been reported for A375 human melanoma cell lines. *In vitro* all-*trans*-RA has been shown to bring about dose- and time-dependent inhibition of the ability of these cells to penetrate matrigel-coated filters using a reconstituted basement membrane invasion assay.[37]

The mechanisms underlying these effects of RA on pigment cells have been studied in detail. In RA-treated B16 cells, changes in the cytoskeleton have been observed. This suggests that these RA-induced modifications in the microtubule and microfilament system could be responsible for the inhibition of migration.[34] Using scanning electron microscopy, it has been shown that RA-treated S91 cells are conspicuously flattened, do not overlap each other and exhibit a markedly increased surface area. Treated cells also appear to be more adherent to the substrate than untreated cells. The change in shape of the cells may account for the increased cell adhesion and, thus, decreased migration.[38]

In A375 human melanoma sublines, the inhibitory effect of RA on tumour cell invasion has been related to a decrease in the secretion of collagenolytic enzymes, with a reduction in the level of type IV collagenase messenger RNA, a decrease in tissue plasminogen activator activity, an increase in the high-affinity metastasis-associated cell surface laminin receptor and decreased expression of a cell-surface receptor for motility factor.[37] Although these results are more closely related to tumour biology, they demonstrate that RA is able to alter cytoskeletal proteins and cell surface receptors of malignant pigment cells. It is also likely that changes in these molecules also occur in normal human melanocytes. A recent study has reported that RA treatment of normal human melanocytes grown in serum-free culture medium alters their morphological appearance; changes include retraction of dendritic processes and increased flattening.[25] Further studies are strongly recommended as an interaction of RA with this different class of molecules could partly explain some of the clinical effects of RA on melanocytes *in vivo*.

Nuclear retinoic acid receptors and pigment cells

The biological effects of RA and its derivatives are likely to be mediated, at least in part, by RA receptors (RARs). In S91 melanoma cells, transcripts complementary to an RAR-γ probe are expressed only at a low level, and their expression is unaffected by RA,[39] as was the expression of RAR-α. On the other hand, the induction of RAR-β is dose-dependent, rapid and insensitive to cycloheximide. Both 13-*cis*-RA and the 3,4,-didehydro derivative of RA also induce expression of RAR-γ, but are only effective at concentrations 100-fold higher than those at which all-*trans*-RA acts.[40]

In another study, similar results have been obtained, demonstrating that S91 melanoma cells constitutively express RAR-α and RAR-γ messenger RNAs. Similar findings have been observed in B16-F1 and K-1735P murine melanoma cells. The level of RAR-β messenger RNA is increased by 10^{-7}–10^{-6} M RA only in B16-F1 and S91 melanoma cells.[19] The mechanism of this induction is not yet known, but the results suggest that binding to cellular RA binding protein is not required for the modulation of RAR messenger RNA levels in this system.[19] Nothing is yet known, however, about the constitutive expression of nuclear retinoic acid receptors in RNAs in normal human melanocytes and their regulation.

Conclusions

The effects of RA on pigment cells are multiple and complex. Although the beneficial action of topical tretinoin on pigmentary abnormalities of photoaged skin is well established, the basic mechanisms underlying these effects are not known. Further studies are needed using pure human melanocyte cultures as well as mixed keratinocyte–melanocyte cultures to evaluate the action of RA on melanogenesis. *In-vivo* studies are also necessary, as it is not always possible to extrapolate from *in-vitro* observations to the *in-vivo* situation, particularly in the field of retinoid pharmacology.

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Addendum

Subsequent to submission of this manuscript, it has been reported that topically applied all-*trans* retinoic (0·5 mg/ml in a solvent consisting of ethanol dimethylsulfoxide and acetone) greatly enhances UV light-induced melanogenesis in lightly pigmented mice (skh: HR-2) which are able to develop a tan. This observation was confirmed in humans. Melanin pigmentation was measured by image analysis in epidermal sheets stained using the Masson Fontana method (Ho KKL, Halliday GM, Barnetson RStC. Topical retinoic acid augments ultraviolet light-induced melanogenesis. *Melanoma Res* 1992; **2:** 41–5). In keeping with these observations, recent data suggest that topical retinoic acid (0·1% for 4 days under occlusion) induces tyrosinase activity in the skin of white subjects *in vivo* (Talwar HS, Griffiths CEM, Fisher GJ, Russman AN, Krach K, Voorhees JT. Topic retinoic acid induces tyrosinase activity in skin of white individuals *in vivo* (Abstract). *J Invest Dermatol* 1992; **98:** 645).

British Journal of Dermatology (1992) 127, Supplement 41, 48–50.

Pigmented lesions as a sign of photodamage

E.HÖLZLE
Department of Dermatology, Heinrich-Heine-University, Düsseldorf, Germany

Summary Chronic exposure to ultraviolet (UV) radiation causes skin changes, known as photodamage. Apart from damage to the connective tissue of the dermis and to keratinocytes, pigmented lesions, known as 'age spots', occur. There are several types, but the most common lesions are the senile lentigines. The main differential diagnoses include reticular seborrhoeic keratosis and lentigo maligna, and histopathological examination is required for exact differentiation. Variants of lentigo senilis with acute onset after intense UV radiation are sunburn freckles. Psoralens- and UVA-induced freckles, and lentigines occur after phototherapy with artificial UV sources or use of sunbeds for cosmetic tanning. Ephelids are common genetically determined pigment spots appearing during childhood in a distinct photodistribution. Exposure to UV radiation also seems to play a role in the manifestation of acquired nevi. Since pigmented lesions in sun-damaged skin represent more than one entity, they differ in their response to treatment. This should be taken into account when the efficacy of topical therapy is evaluated.

Photodamage induced by chronic exposure to ultraviolet (UV) radiation involves different sections of the skin. Damage to the fibroblasts results in changes in the various constituents of dermal connective tissue. Keratinocyte damage may lead to precancerous changes and non-melanoma skin cancers. The melanocytes are involved in pigmentary lesions, and damage to the epidermal Langerhans cells may result in a modulation of the immune function.

In addition to loss of elasticity and changes in the skin texture, alterations in skin colour and the appearance of pigmented spots are perceived to be clinical signs of ageing. Pigmented lesions of photodamaged skin are commonly designated as 'age spots'. The different types of pigmented lesions and their histology and pathogenesis are reviewed.

Types of pigmented lesions

Ephelids (freckles)

Ephelids are multiple pigmented spots measuring a few millimetres in diameter. They are genetically determined and vary in their colour intensity according to sun exposure.[1] They follow a distinct photodistribution and are most common in Caucasians of skin types I and II. Ephelids appear in childhood, increase in number in adults, and seem to regress during old age. The most common sites are the nose, cheeks, shoulders and extensor aspects of the arms. It is assumed that a somatic mutation of epidermal melanocytes leads to increased melanin production.

The epidermis remains histopathologically unchanged, but melanocytes and basal keratinocytes contain increased numbers of melanosomes, although the number of melanocytes appears to be unaltered.[2] The melanosomes within ephelids ultrastructurally resemble the large melanosomes of dark-skinned people.[3]

Lentigo senilis

Lentigo senilis, the prototype of the so-called 'age spot',[4] represents a pigmented macule in chronically UV-exposed skin. It appears mainly in Caucasians, and in Mongolians, between the fourth and sixth decades of life. The face and extensor aspects of the forearms and the backs of the hands are predominantly involved. The lesions, which are sharply but irregularly outlined, vary in colour from yellow–brown to dark brown, the colour being independent of the extent of UV exposure. The size may range from a few millimetres to several centimetres in diameter. Lentigines seniles are usually numerous and they may be accompanied by adjacent patchy hypopigmentation, which results in the skin having a mottled appearance.

Epidermal hyperplasia is combined with hyperpigmentation and actinic damage of the dermis, and there is irregular elongation of rete ridges with hyperpigmentation predominantly at the tips. Melanocytes remain unchanged in number, in relation to length of basal

Correspondence: Professor Dr E.Hölzle, Hautklinik der Heinrich-Heine-Universität, Moorenstrasse 5, 4000 Düsseldorf 1, Germany.

membrane, although some authors have reported increased numbers of melanocytes.[5] Ultrastructural studies have demonstrated that the melanocytes show signs of increased melanization.[6]

Acute UV-induced lentigines

Included in this category are sunburn freckles, psoralen- and UVA-induced lentigines,[5,7] and freckles following intense exposure to UV radiation associated with phototherapy or the use of sunbeds.[8] When induced experimentally by UVB irradiation these lentigines appeared within 2–3 weeks.[9] Histopathologically the lesions resemble lentigo senilis.

Reticular seborrhoeic keratoses

These lesions are sometimes clinically indistinguishable from lentigo senilis. They appear usually in small numbers in sun-exposed areas of skin in middle-aged or older patients. The macules are sharply outlined, frequently oval in shape, and sometimes irregularly formed. They vary in colour from light to dark brown and, in contrast to the usual seborrhoeic keratoses, exhibit a smooth surface.[10] It is a matter of debate whether or not a reticular seborrhoeic keratosis can evolve from lentigo senilis.[11]

In reticular seborrhoeic keratosis, there is little orthohyperkeratosis, and reticulated hyperplasia of the epidermis with inclusion of pseudo-horn cysts. The melanin content of basal cell layers is increased, the papillary dermis is thickened, and there are signs of actinic damage in the corium.[12]

Lentigo maligna

Lentigo maligna is also called melanotic freckle of Hutchinson[13] or melanosis circumscripta praecancerosa (Dubreuilh).[14] This type of lesion is of great clinical significance, since it represents a malignant melanoma *in situ*.[15] It is important to differentiate between lentigo maligna and either lentigo senilis or reticular seborrhoeic keratosis. Fair-skinned subjects are more prone to develop lentigo maligna, sites of predilection being the face, particularly the cheeks and nose, extensor aspects of the arms and, in women, the lower legs. Patients are mainly beyond the fifth decade of life, and the female:male ratio of incidence is 2:1.

Typical clinical features include variation in colour, ranging from light brown to black, fuzziness of the borders and its large size, measuring up to several centimetres in diameter. In amelanotic variants, diagnosis is entirely dependent on histopathology.[16] The skin surface remains unchanged; effacement of dermatoglyphics and nodular growth are signs of invasive malignant melanoma.

Histological examination shows that the skin possesses the characteristic features of sun damage with actinic elastosis and a thin epidermis with loss of rete ridges. Atypical melanocytes are increased in number and dispersed as solitary units or small nests at all levels of the epidermis. In the upper corium, infiltrates of lymphocytes and pigment in macrophages are present.

Conclusions

Spotty pigmented lesions are an important sign of photodamage. The so-called 'age spot', however, represents different entities, the most common variant being the lentigo senilis. The main differential diagnoses include reticular seborrhoeic keratosis and lentigo maligna. The latter represents malignant melanoma *in situ* and requires accurate diagnosis and treatment. Other benign pigmented lesions, which are genetically determined and which appear during childhood and young adulthood, follow a distinct photodistribution. These include ephelids and, to a certain extent, acquired nevi.

Since photo-induced pigmentary lesions represent different entities, they also differ in their pathogenetic mechanisms and, consequently, will vary in their response to treatment. Clinical studies on the efficacy of topical treatment of photodamage should take these differences into account.

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British Journal of Dermatology (1992) 127, Supplement 41, 51–53.

Experience with tretinoin therapy in temperate regions

R.CAPUTO, M.MONTI, C.RIGONI, S.PINELLI, S.MOTTA AND M.BARBARESCHI
First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy

Summary

In a previously reported study on the anti-photoageing effects of topical tretinoin, the following regimen produced good patient compliance: 0·01% for 1 month, 0·025% for 1 month; and 0·05% for 4 months. The majority of patients (60/89) enrolled in the initial study continued to apply the cream to the face, and a further 140 patients were enrolled for a long-term study (mean duration 2 years). The prolonged study showed that 91·4% of patients used tretinoin in an attempt to slow down skin ageing, and 8·6% sought subjective skin benefits. Of the 163 patients who completed the study, 58·8% sought an improvement of wrinkles, 30·1% skin trophism and 14·7% reduced pigmentation. The product was used throughout the year by 66·9% of patients, but 8·0% stopped using it during the summer. A daytime moisturizing cream was required by 77·9% of patients, and 82·8% used a sunscreen in the summer. Tretinoin was applied to other areas of the body by 63·8% of patients.

Photodamage is responsible for most of the visible signs of ageing skin and for histological tissue changes, such as epidermal atrophy and dysplasia, abnormal melanocytic activity and dermal elastosis. The changes caused by chronic exposure to the sun may be partially reversed using topical tretinoin (all-*trans*-retinoic acid).[1–3]

The anti-photoageing efficacy of tropical tretinoin was originally evaluated in an open clinical trial involving 89 patients. Efficacy was assessed clinically and photographically together with the use of replica analysis.[4] The study included patients of Mediterranean origin, and data on tolerability and compliance were obtained. During the 6-month trial, the following regimen was employed in order to increase product tolerability and patient compliance: 0·01% tretinoin was applied once daily for the first month, 0·025% tretinoin was applied for the second month, and 0·05% tretinoin was applied from the third to sixth month of treatment. At the end of the 6-months' treatment, most of the clinical symptoms of photoageing (i.e. coarse wrinkling, fine wrinkling, skin thinning, mottled hyperpigmentation and laxity), except for xerosis, were improved. Tolerability was considered excellent in 51·4% of patients, good in 44·4% and fair in 4·2%, and compliance was excellent in 47·0% of patients, good in 48·5% and fair in 4·5%. Only five (5·6%) of the 89 patients enrolled dropped out for reasons related to the use of tretinoin.

The aim of this follow-up study was to evaluate the efficacy and safety of tretinoin in 200 patients (60 from the first trial and 140 new patients) over a period of 2 years. Each patient was free to choose the concentration of tretinoin within the range 0·01–0.05% and the application regimen according to personal experience. The study also aimed to gain information about the preferred concentration of tretinoin and modality of application, and the patients' attitude to the use of a pharmaceutical product for a cosmetic purpose.

Patients and methods

Patients

A total of 200 healthy female patients ranging in age from 33 to 67 years (mean age 50·1 years) were recruited to the trial in September 1988. All subjects had mild or moderate photodamage.

Treatment

At enrolment, the investigator gave each patient three creams each containing a different concentration of tretinoin (0·01%, 0·025% and 0·05%). The investigator suggested that the cream containing the lowest concentration should be used once daily at bedtime for the first month, the medium-concentration cream during the second month and, thereafter, the highest-concentration cream. The treatment was continued for 2 years. In the event of skin irritation, the patient could return to a lower concentration.

Correspondence: Ruggero Caputo, MD, First Department of Dermatology and Paediatric Dermatology, University of Milan, Via Pace 9, 20122 Milan, Italy.

Clinical evaluations

Prior to treatment the patients were clinically evaluated and the overall severity of photodamage was graded on a 9-point scale as follows: 1–3 = normal to mild; 4–6 = mild to moderate; 7–9 = moderate to severe photodamage. In addition, individual signs of photodamage, including coarse wrinkling, fine wrinkling, skin thinning, mottled hyperpigmentation, laxity and xerosis, were graded on a 9-point scale, and colour photographs obtained under standardized conditions were taken at baseline and at the end of treatment. Silicon rubber skin replicas from the lateral periorbital area were taken for profilometric studies.[5]

At 3-month intervals, the patients returned to the clinic for evaluation using a questionnaire. The following information was obtained: the reason for using tretinoin; the concentration of tretinoin used; the patients' self-assessment of global improvement; the patients' self-assessment of the main results obtained by the use of tretinoin cream; the period of continuous application of tretinoin cream; the need for treatment of dry skin; the use of a sunscreen during the summer; and whether or not the tretinoin cream had been applied to areas other than the face.

Results

Over the 2-year period of the open study, 37 (18·5%) patients withdrew from treatment: twelve for reasons related to the use of tretinoin and 25 patients for unrelated reasons. At the end of the treatment period, the results from 163 patients were assessed. The main results of the guided questionnaire conducted at 3-monthly intervals were summarized as follows: 149 (91·4%) patients stated that the main reason for their using tretinoin cream was to retard skin ageing, and 14 (8·6%) patients used the cream for a subjective skin benefit. Sixty-seven (41·1%) patients used the cream containing 0·025% tretinoin, 63 (38·7%) patients used 0·05% tretinoin and 15 (9·2%) patients used 0·01% tretinoin throughout the trial, and the remaining 18 (11·0%) patients used the cream at various concentrations. At the end of treatment, self-assessment of global improvement was considered to be slight by 49 (30·1%) patients, moderate by 93 (57·1%) patients and marked by 21 (12·9%) patients. The main change occurring following the use of the tretinoin cream was a reduction of wrinkles, which was reported by 90 (58·8%) patients. In addition, 49 (30·1%) patients reported an improvement in skin texture and 24 (14·7%) patients experienced a decrease in lentigines and pigmentation. The cream was used once daily throughout the trial period by 109 (66·9%) patients, 13 (8·0%) patients stopped applying the cream during the summer, and 41 (25·2%) patients only applied the product erratically. A moisturizing cream applied during the day was required by 127 (77·9%) patients to counteract dryness induced by the topical tretinoin, and 135 (82·8%) patients used a sunscreen during the summer. The cream was spontaneously applied to sites other than the face, such as elbows, knees, hands and feet, by 104 (63·8%) patients; the reason for applying the product to other areas was the improvement of facial skin texture.

Discussion

The 2-year open clinical trial, which was undertaken to obtain an understanding of how better to manage the administration of tretinoin in a Mediterranean population, allowed several conclusions to be drawn.

The majority of the patients in the study used tretinoin to retard skin ageing or to reduce solar damage; however, 9·0% of patients were not interested in ageing problems. These patients were quite sceptical about the anti-photoageing properties of the product, but they stated that the reason for their using it was that they thought that the benefit to the skin was superior to that of any other cosmetic face treatments.

It was observed that 41·1% of patients preferred the cream containing 0·025% tretinoin, compared to 38·7% who used the cream containing 0·05%, and 9·2% of patients who could not tolerate a concentration greater than 0·01% tretinoin. Despite the fact that sensitive phototype I patients were not enrolled in the present trial, more than 50% of the patients used the lower tretinoin concentrations (0·025% and 0·01%). Thus it can be concluded that the southern European women treated in the trial were not attracted by the argument—'higher concentration, higher activity'. Patients were not prone to minimal retinoid dermatitis.

As in the previous trial,[4] patients were more optimistic about the improvement which they obtained than were the investigators. It is noteworthy that about 45% of patients studied considered that the main result obtained following the use of tretinoin was not a reduction in wrinkles but other modifications, such as improvement in skin texture (30·1% of patients) or decrease in pigmentation (14·7% of patients). These data reflect the wide range of activity that this topical product had in a variety of skin types; the reduction of wrinkles was only one of the results obtained following application of tretinoic acid.

Another interesting result of the study was the finding that more than half (67·0%) of the patients studied applied the tretinoin-containing cream to other sites, including elbows, knees, hands and feet. In the patients' opinion, the skin at these sites had become thicker with age, and the tretinoin was able to restore its softness.

In conclusion, the trial demonstrated that patient compliance was good. The patients were enthusiastic about the use of tretinoin for cosmetic purposes, and they appreciated the opportunity to personalize the treatment according to skin response, and to use tretinoin on areas other than the face. Dermatologists should therefore bear in mind the response of the patients to the treatment.

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Contributors

M.BARBARESCHI *First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy*

R.CAPUTO *First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy*

C.EDWARDS *Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff, UK*

L.J.FINKEL *Dermatopharmacology Unit, Department of Dermatology, University of Michigan Medical Center, Ann Arbor, MI, USA*

G.J.FISHER *Dermatopharmacology Unit, Department of Dermatology, University of Michigan Medical Center, Ann Arbor, MI, USA*

B.A.GILCHREST *USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA, USA*

C.E.M.GRIFFITHS *Dermatopharmacology Unit, Department of Dermatology, University of Michigan Medical Center, Ann Arbor, MI, USA*

E.HÖLZLE *Department of Dermatology, Heinrich-Heine-University, Düsseldorf, Germany*

R.MARKS *Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff, UK*

M.MONTI *First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy*

S.MOTTA *First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy*

J.-P.ORTONNE *Service de Dermatologie, Hôpital Pasteur, Nice, France*

S.PINELLI *First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy*

C.RIGONI *First Department of Dermatology and Paediatric Dermatology, University of Milan, and Ospedale Maggiore (IRCCS), Milan, Italy*

R.RUSSELL JONES *St John's Dermatology Centre, St Thomas's Hospital, London, UK*

E.G.THORNE *R.W.Johnson Pharmaceutical Research Institute, Raritan, NJ, USA*

J.J.VOORHEES *Dermatopharmacology Unit, Department of Dermatology, University of Michigan Medical Center, Ann Arbor, MI, USA*

M.YAAR *Department of Dermatology, Boston University School of Medicine, Boston, MA, USA*

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British Journal of Dermatology

Volume 127

July–December 1992

Published for the
British Association of Dermatologists
by Blackwell Scientific Publications
OXFORD LONDON EDINBURGH BOSTON
MELBOURNE PARIS BERLIN VIENNA

British Journal of Dermatology

The *British Journal of Dermatology* is owned by and is the official organ of the British Association of Dermatologists. It is also the official organ of the Nederlandse Vereniging voor Dermatologie en Venereologie (Netherlands Association for Dermatology and Venereology).

EDITOR
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Department of Dermatology, Leicester Royal Infirmary, Leicester LE1 5WW

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The British Journal of Dermatology is published monthly and the subscription price for 1992 is £135.00 (U.K.), £143.00 (overseas except North America), $245.00 (U.S.A. and Canada), in all cases post free. Subscriptions should be addressed to Journal Subscription Dept., Marston Book Services, P.O. Box 87, Oxford, U.K. Tel 0865 791155, Telex 837515 Mardis G, Fax 0865 791927.

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Printed in Great Britain at The Alden Press, Oxford

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British Journal of Dermatology (1992) 127, 1–4.

Distribution of complement regulators (CD46, CD55 and CD59) in skin appendages, and in benign and malignant skin neoplasms

K.SAYAMA, S.SHIRAISHI AND Y.MIKI
Department of Dermatology, Ehime University School of Medicine, Shigenobucho, Ehime, Japan

Accepted for publication 26 January 1992

Summary Immunohistochemical studies were performed to establish the distribution of membrane cofactor protein (MCP; CD46), decay-accelerating (DAF; CD55) and homologous restriction factor (HRF20; CD59), in normal skin appendages, and in benign and malignant skin neoplasms. At least two of these regulators were detected on normal eccrine glands, apocrine glands and sebaceous glands. They were also found in cellular naevi (CN), seborrhoeic keratoses (SK), basal cell carcinoma (BCC), Bowen's disease (BD), squamous cell carcinoma (SCC) and Paget's disease (PD). Although there were slight differences in their distribution, these regulators were found in all the cells examined, indicating that they are essential factors in human skin as well as other organs, and in neoplasms, in preventing autologous complement attack.

Activation of complement may lead to the deposition of C3b/C4b on the target organ, and release of anaphylatoxins or formation of the membrane attack complex on the target cell membrane, resulting in tissue destruction. Complement plays an important role in a number of dermatological disorders. Complement deficiency may be associated with skin disease, and complement is important in the pathogenesis of vasculitis and bullous pemphigoid.[1–4] In normal conditions, cells are protected from autologous complement attack by the presence on cell membranes of regulatory proteins such as MCP,[5] DAF,[6] homologous restriction factor (HRF),[7,8] and HRF20.[9] These membrane proteins regulate different stages of complement activitation. MCP is a potent factor I-co-factor, DAF is an accelerator of the decay of C3/C5 convertases, and HRF and HRF20 regulate the membrane attack complex.

These complement regulators have been shown to have homologous restriction.[5,7,9] The complement system has its own mechanisms to recognize self and non-self by these regulators. When the regulators are absent from the cell membrane or not compatible with the complement in plasma, the cell will be attacked. A deficiency of DAF and HRF20 on erythrocytes has been demonstrated in paroxysmal nocturnal haemaglobinuria,[10] and these cells are susceptible to homologous complement attack. Neoplastic cells are believed to be able to escape from immune surveillance, including the complement system. It has been reported that some neoplastic cells are protected from complement-mediated cytotoxicity by these complement regulators.[11,12]

Although the distribution and characterization of MCP, DAF and HRF20 on normal human epidermis and endothelial cells has been reported,[13–15] the distribution on normal skin appendages and neoplastic cells has not been studied. In this study, the distribution of these regulators on normal skin appendages and skin neoplasms is described.

Methods

Immunohistochemical studies were performed as previously described.[13–15] Excised specimens of skin neoplasms with surrounding normal skin were mounted in OCT compound (Miles Inc., Elkhart, IN, U.S.A.), and 4-μm sections were cut and fixed with acetone/chloroform (1:1) at −20°C for 9 min. Staining with a PAP kit (Ortho Diagnostic Systems Inc., Raritan, NJ, U.S.A.) was performed according to the instructions provided by the manufacturer. After blocking with hydrogen peroxide and normal rabbit serum, the monoclonal antibodies anti-MCP (M-75 and M-177,[16] gifts from Dr T.Seya of the Centre for Adult Disease, Osaka, Japan), anti-DAF (IA10,[10] a gift from Dr T.Kinoshita of Osaka University, Osaka, Japan) and anti-HRF20 (IF5,[17] a gift from

Correspondence: Dr Koji Sayama, Department of Dermatology, Ehime University School of Medicine, Shigenobucho, Onsengun, Ehime 791–02, Japan.

Dr H.Okada of Nagoya City University, Nagoya, Japan) were added to the sections and incubated in a moist chamber for 1 h at room temperature. Second antibody and peroxidase-antiperoxidase (PAP) were reacted with the specimens, sequentially. Some specimens were counterstained with haematoxylin.

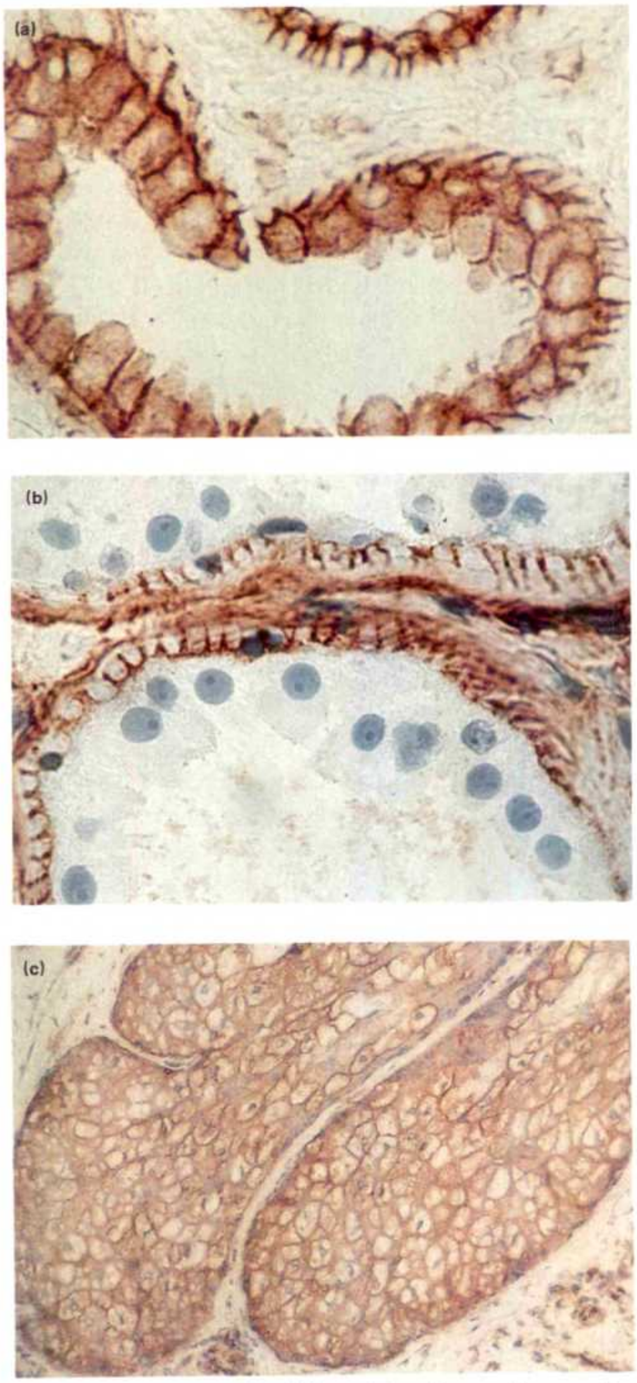

Figure 1. Photomicrographs of the staining of normal human skin appendages. (a) Apocrine glands with anti-MCP (PAP, ×400). (b) Apocrine glands with anti-DAF. (c) Sebaceous glands with anti-HRF20 (×200).

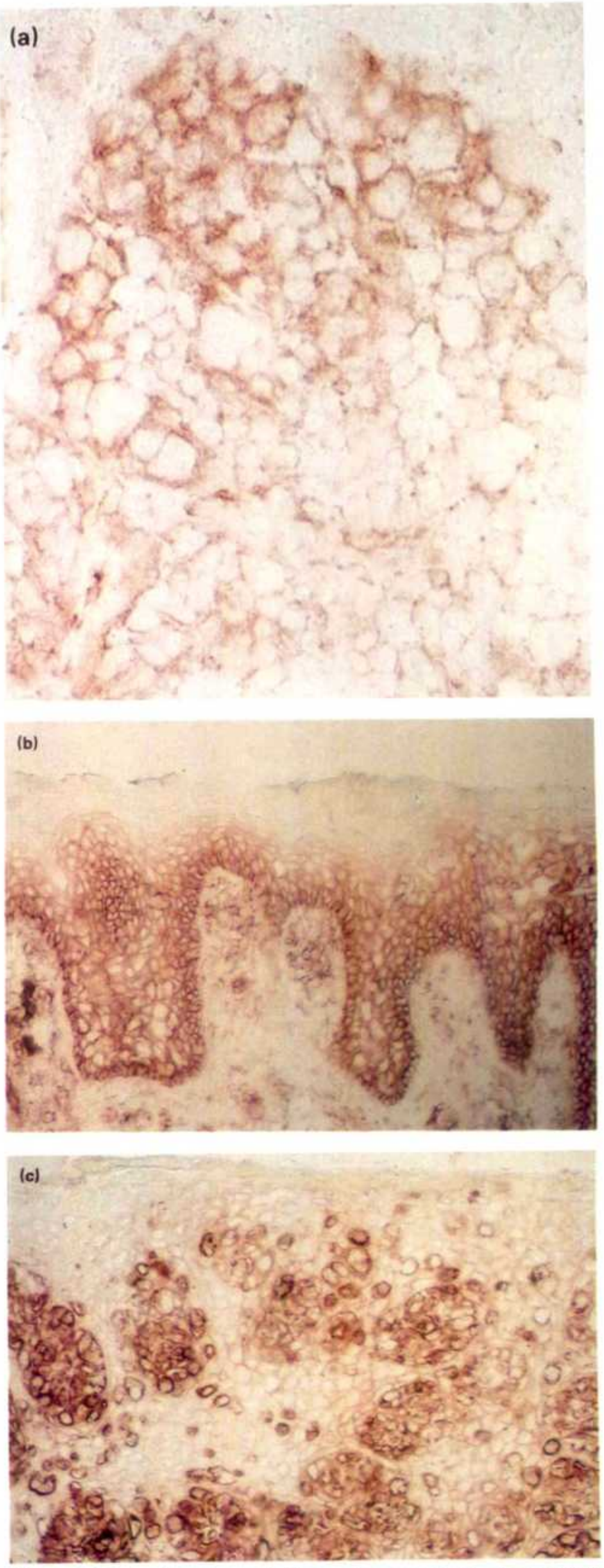

Figure 2. Photomicrographs of skin neoplasms stained with anti-MCP. (a) CN (PAP, ×400). (b) BD (×100). (c) PD (×150).

Results

Distribution of MCP, DAF and HRF20 in normal skin appendages

Although MCP and HRF20 were expressed on eccrine secretory cells, apocrine secretory cells, apocrine myoepithelial cells and sebaceous glands, DAF was expressed only on apocrine myoepithelial cells (Fig. 1). Eccrine myoepithelial cells were not clearly distinguished by this staining procedure. The results from previous reports[13–15] of staining of skin appendages, epidermis, endothelial cells and elastic fibres in normal skin are summarized in Table 1.

Expression of MCP, DAF and HRF20 on skin neoplasms

Table 2 summarizes the results of the staining of cells in CN, SK, BCC, BD, SCC and PD with anti-MCP, DAF and HRF20. Photomicrographs of sections stained with anti-MCP are shown in Figure 2. MCP and HRF20 were expressed by almost all neoplastic cells examined, but DAF was not detected on CN, PD and some BCC cells. In SK, BD and SCC, gradual loss of labelling with MCP and HRF20 occurred as the cells became keratinized, and there was a gradual increase in labelling with DAF. Within the cell nests of BCC, however, there were no differences in labelling.

Table 1. Distribution of MCP, DAF and HRF20 in normal human skin

	MCP (CD46)	DAF (CD55)	HRF20 (CD59)
Epidermis*	Predominant in basal layer	Predominant in granular layer	Predominant in basal layer
Endothelial cell*	+	+	+
Eccrine gland†			
Secretory cell	+	−	+
Apocrine gland			
Secretory cell	+	−	+
Myoepithelial cell	+	+	+
Sebaceous gland	+	−	+
Elastic fibre*	−	+	−

*References 13–15.
†Eccrine myoepithelial cells were not clearly distinguishable by this staining procedure

Table 2. Expression of MCP, DAF and HRF20 on skin neoplasms

	MCP (CD46)	DAF (CD55)	HRF20 (CD59)
Benign			
CN	7/7	0/7	7/7
SK	5/5	5/5	5/5
Malignant			
BCC	5/5	2/5	4/5
BD	5/5	5/5	5/5
SCC	5/5	5/5	5/5
PD	5/5	0/5	5/5

CN, cellular naevus; SK, seborrhoeic keratosis; BCC, basal cell carcinoma; BD, Bowens's disease; SCC, squamous cell carcinoma, PD, Paget's disease.

Discussion

In previous studies,[13–15] MCP, DAF, and HRF20 have been demonstrated on normal keratinocytes and endothelial cells. These complement regulators are considered to be widely distributed throughout the body to maintain homeostasis in the presence of the complement system. DAF has been reported to be present on erythrocytes, leucocytes, platelets, endothelial cells, cornea, gastrointestinal mucosa, kidney, bladder, uterus and lung, and soluble DAF is found in plasma, tears, saliva and urine.[17] HRF20 has been reported to be present on erythrocytes, leucocytes, endothelial cells, the Schwann sheath of peripheral nerve fibres, ependymal cells, salivary glands, bronchial epithelium and renal tubules.[18] The distribution pattern of HRF20 differs somewhat from that of DAF. MCP is also believed to be widely distributed in the body, although *in vivo* study of its expression is still limited, except in leucocytes, platelets and skin.[13,19]

In this study, we have shown that these regulators are widely distributed in normal human skin including skin appendages. The distribution of MCP was almost the same as that of HRF20, but differed from that of DAF. At least one of these regulators was present in the epidermis, endothelial cells, appendages and elastic fibres of normal human skin. Variation in their distribution and function suggests that they may compensate for each other in order to protect the cells from complement attack.

Benign and malignant neoplastic cells also demonstrated at least two of the regulators. PD and normal apocrine secretory cells did not express DAF. This lends support to the view that PD cells originate from apocrine glands. The absence of DAF on some BCCs may be related to the finding that DAF was barely detectable on the basal layer of normal epidermis. There are gradual changes of the labelling of MCP, DAF and HRF20 in normal epidermis as the cells migrate from the basal cell layer to the stratum corneum[13–15] (Table 1). The gradual changes of labelling of these regulators in SK, BD and

SCC as the cells keratinize are similar to those found in normal epidermis.

In conclusion, MCP, DAF and HRF20 are widely distributed throughout normal human skin, including appendages, and also in benign and malignant neoplasms.

Acknowledgment

This work was supported in part by a 1991 grant of the Japanese Ministry of Health and Welfare.

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A comparison of the efficacy and relapse rates of narrowband UVB (TL-01) monotherapy vs. etretinate (re-TL-01) vs. etretinate-PUVA (re-PUVA) in the treatment of psoriasis patients

CATHY GREEN, T.LAKSHMIPATHI, B.E.JOHNSON AND J.FERGUSON

The Photobiology Unit, Department of Dermatology, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.

Accepted for publication 13 February 1992

Summary Forty-five patients with extensive chronic plaque or guttate psoriasis were treated with either narrowband (TL-01) phototherapy, etretinate TL-01 combination therapy (re-TL-01) or etretinate and PUVA (re-PUVA) (15 patients in each group). Re-PUVA was the most effective therapy with 100% satisfactory clearance rate. TL-01 monotherapy had an 80% success rate; the relapse rate compared favourably with re-PUVA (50% in remission after 6 months). In the etretinate-TL-01 group, there was a 93% success rate and a one-third reduction in the total irradiation dose (8·0 J/cm^2 vs. 12·7 J/cm^2) but the relapse rate was higher, only 33% remaining in remission after 6 months.

Whilst conventional UVB phototherapy (290–320 nm) and photochemotherapy (PUVA) are established treatments for patients with psoriasis, efforts continue to refine their efficacy and safety.

Debate has focused on the risk of developing cutaneous carcinoma as a consequence of PUVA therapy.[1,2] In the absence of other risk factors,[1] this would appear to be related to the total cumulative UVA dosage.[2] UVB phototherapy also carries a potential risk of carcinogenesis[3,4] although a clinical study has failed to demonstrate this.[5]

Knowledge that the oral retinoids are effective in the treatment of psoriasis and may have an anticancer effect[6] has led to the study of these drugs in combination with phototherapy (re-UVB)[7] or photochemotherapy (re-PUVA).[8,9] Treatment with etretinate alone followed by concomitant PUVA allows clearance with, on average, a one-third reduction of the total cumulative UVA exposure dose required with PUVA alone. A similar reduction in total UVB dose is also achieved by the addition of etretinate to UVB phototherapy.[7]

The use of conventional broadband UVB sources is limited by burning.[10] Recently, a new UVB lamp has been developed (Philips TL-01),[11] which is virtually a monochromatic source, with a peak narrowband emission at 312 nm and a minor peak at 305 nm, thereby omitting the shorter more erythemogenic and carcinogenic wavelengths. This lamp is clinically useful in the treatment of psoriasis.[11,12] Although a preliminary study suggests that the emission may have a lower carcinogenic potential for mouse skin than that of the conventional broadband UVB sources,[11] other workers disagree.[13]

While etretinate is not phototoxic *in vitro* its principal metabolite Ro 10-1670 is. In a study of nine patients taking etretinate[14] who were phototested, two showed increased sensitivity most marked on monochromator testing between 305 and 320 ± 5 nm, making a combination study of etretinate with the TL-01 lamp of particular interest. Whether this presumed phototoxicity has a therapeutic effect, as occurs with psoralens, is uncertain.

We decided, therefore, to compare the effects of TL-01 monotherapy with the combination of etretinate and TL-01 (re-TL-01). Re-PUVA, a proven successful combination therapy, is complicated by the requirement for a psoralen. This complication can be avoided by re-TL-01 treatment and for this reason a second limb was set up to compare re-TL-01 and re-PUVA.

Methods

Forty-five adult patients (25 males, 20 females) with extensive chronic plaque or guttate psoriasis were entered into the study. Patients were included if they

Correspondence: Dr C.Green, The Photobiology Unit, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.

either (i) failed to respond to UVB or PUVA previously, (ii) had experienced rapid relapse following UVB or PUVA, or (iii) had a high cumulative PUVA dose (>1000 J/cm^2). Exclusion criteria included co-existent hepatic or renal malfunction, PUVA, methotrexate, or retinoid therapy over the previous 2 months and a history of ischaemic heart disease or hyperlipidaemia. Female patients of child-bearing age not protected by contraception were also excluded from the study, as were patients with a history of cutaneous malignancy. The study was approved by the hospital ethical committee, and written informed consent was obtained from all patients. At the initial visit, using a sealed code kept in the pharmacy, patients were randomized to receive one of three therapies: re-PUVA, re-TL-01 or TL-01 monotherapy.

Routine investigations performed on each patient included full blood count and film, erythrocyte sedimentation rate, urea and electrolytes, liver function studies, anti-Ro and anti-La antibodies, antinuclear factor and fasting lipids (cholesterol and triglycerides). Whole body clinical examination was performed for evidence of cutaneous malignancy. The minimal erythema dose (MED) was determined for those patients on TL-01 monotherapy or re-TL-01 and the minimal phototoxic dose (MPD) for the re-PUVA group (PUVA source: Waldman PUVA 6000 cubicle; 62 Sylvania tubes, maximal emission at 365 nm, irradiance 8·0 mW/cm^2). The MED or MPD was determined before treatment, and after 2 weeks of etretinate therapy to assess any change.

For the duration of the study patients were permitted to use only 50/50 liquid paraffin/white soft paraffin as an emollient, as the use of other agents such as yellow soft paraffin has been shown to impair transmission of ultraviolet radiation.[15]

Table 1 lists the three treatment schedules. The TL-01 group received treatment thrice weekly, starting with 70% of the MED. Thereafter, incremental increases of 40% were chosen to achieve slight erythema with each subsequent dose.[12] If marked erythema or burning (defined as severe painful erythema) resulted, the dosage was adjusted (Table 1). Once a satisfactory response was achieved (the patient's skin was clear or there was minimal residual activity), treatment was continued for a further 2 weeks. For the re-TL-01 group, etretinate was used at a dose of 1 mg/kg body weight unless adverse events necessitated a dose reduction. Pretreatment with etretinate for 2 weeks was followed by TL-01 phototherapy thrice weekly in combination with etretinate.

In the re-PUVA group, pretreatment with etretinate (1 mg/kg daily) for 2 weeks was followed by a combination of etretinate and PUVA (8-methoxypsoralen 0·6 mg/kg twice weekly, or in those with upper gastrointestinal problems such as nausea, 5-methoxypsoralen 1·2 mg/kg). The initial PUVA dose was 0·5 J/cm^2 below the MPD, with increments of 0·5–1·0 J/cm^2 weekly. Again, once a satisfactory response was achieved, treatment continued for a further 2 weeks.

Table 1. Three treatment schedules

A. TL-01 phototherapy
1. Determine MED
2. First treatment. 70% of MED
    If no erythema: increase by 40%
    If slight erythema: increase by 20%
    If more than slight erythema: same dose
    Mild burn· decrease by 50%
    Severe burn: omit dose
Aim for mild erythema response with each subsequent dose
3. Treatment three times weekly
4. Once 'satisfactory' response—continue treatment for 2 weeks

B. Re-TL-01
1 Determine MED
2. Etretinate 1 mg/kg body weight/day for 2 weeks
3. MED repeated
4. re-TL-01 as in Schedule A
5. re-TL-01 continued for 2 weeks after satisfactory response

C. Re-PUVA
1 Determine MPD
2. Etretinate 1 mg/kg body weight/day for 2 weeks
3. MPD repeated
4. re-PUVA
    8-methoxypsoralen 0·6 mg/kg
    First treatment 0·5 J/cm^2 below MPD
    Treatments twice weekly
    Increments of 0·5–1·0 J/cm^2 weekly
5. Once satisfactory response, re-PUVA continued for 2 weeks

Patients were assessed for clinical improvement and adverse events at 2-week intervals. Once therapy was stopped, patients were reassessed every 2 weeks for 1 month, and monthly thereafter. A relapse was defined as a return of psoriasis to 50% or more of that at study entry.

Results

Routine laboratory investigations before and after treatment were normal in all patients, excepting minor lipid abnormalities in four patients, detailed below.

Figures 1 and 2 provide a graphic representation of response to therapy and relapse rates in the three different treatment groups. Exposure dosages and

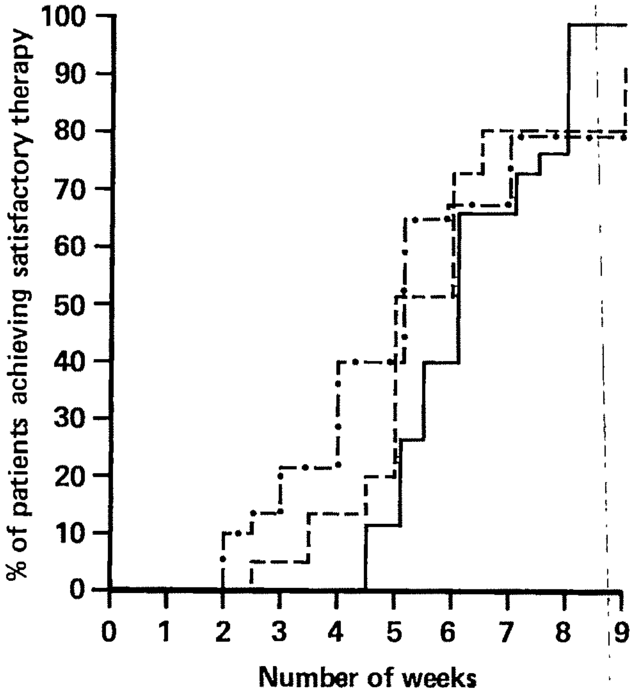

Figure 1. Comparison of response to therapy in the three treatment groups. —●—, TL-01; – – –, re-TL-01; ——, re-PUVA.

duration of therapy are detailed in Table 2 and adverse effects in Table 3.

TL-01 group

The response to treatment was rapid, with a mean duration of 4·5 weeks (range 1·6–9·0) until satisfactory clearance occurred. Twelve of 15 patients achieved a satisfactory response, with three being withdrawn due to problems with burning and pruritus. The mean cumulative exposure dosage was 12·7 J/cm^2 (range 4·2–24·4). Fifty per cent of patients remained in remission 6 months after stopping treatment.

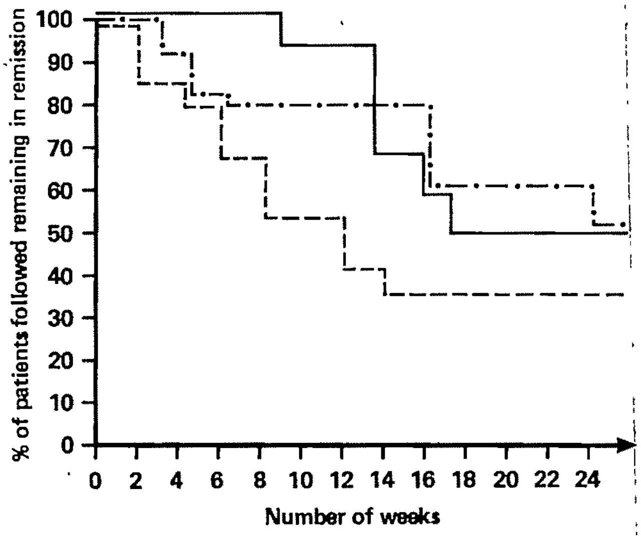

Figure 2. Comparison of relapse rates in the three treatment groups. —●—, TL-01; – – –, re-TL-01; ——, re-PUVA.

Re-TL-01 group

Fourteen of this group of 15 patients achieved a satisfactory response in an average of 6 weeks (range 2·6–9·0) with a total exposure dose of 8·0 J/cm^2 (range 1·0–22·5). One patient was withdrawn because of pruritus and burning and another had etretinate-induced reversible diffuse hair loss. Another developed hypercholesterolaemia whilst on etretinate, but the cholesterol returned to normal on stopping therapy. Thirty per cent of patients remained in remission at 6 months follow-up.

Re-PUVA group

All patients in this group achieved a satisfactory response to therapy in a mean of 6·1 weeks (range 4·5–8·0) and with a mean total exposure dose of 33·0 J/cm^2 (range 10·0–57·0). One patient experienced severe nausea with 8-methoxypsoralen which improved on changing to 5-methoxypsoralen. Another experienced diffuse hair loss on etretinate and three developed reversible hypertriglyceridaemia due to etretinate. As in the TL-01 group, 50% of patients remained in remission at 6 months.

Summary of results

The results are summarized in Table 4. re-PUVA was the most effective therapy with 100% satisfactory clearance rate. Interestingly, TL-01 monotherapy compared favourably with re-PUVA, the relapse rate being the same. As expected, the addition of etretinate to TL-01 therapy provided a one-third total irradiation dose reduction.

Results of MED and MPD testing

In 12 patients in the re-TL-01 group and 11 in the re-PUVA group, we were able to compare MED and MPD values before and after 2 weeks of etretinate therapy. The results, shown in Figure 3, failed to demonstrate a statistically significant phototoxic etretinate effect.

Table 2. Duration of therapy required to achieve a satisfactory response

Patients achieving satisfactory response	Mean number of treatments (range)	Mean duration of therapy weeks (range)	Mean total exposure dose J/cm^2 (range)
TL-01 ($n=12$)	14 (5–27)	4·5 (1·6–9·0)	12·7 (4·2–24·4)
re-TL-01 ($n=14$)	15 (2–21)	6·0 (2·6–9·0)	8·0 (1·0–22·5)
re-PUVA ($n=15$)	9 (5–12)	6·1 (4·5–8·0)	33·0 (10·0–57·0)

Table 3. Side-effects of therapy

	TL-01	re-TL-01	re-PUVA
Severe burn	3	1	0
Pruritus	3	1	0
Nausea	—	0	1
Diffuse hair loss	—	1	1
Raised fasting cholesterol	—	1	0
Raised fasting triglyceride	—	0	3

Discussion

The TL-01 lamp manufactured by Philips excludes the shorter erythemogenic wavelengths emitted by conventional broadband UVB sources and therefore may be expected to produce fewer problems from burning. In animal studies, the action spectrum for UV-carcinogenesis is thought to parallel that for erythema;[16] the source may also have the theoretical advantage of being less carcinogenic than the conventional broadband type,[11] although this is under debate.[13]

This study is the first combining etretinate and the new TL-01 lamp. As previously,[12] TL-01 monotherapy is effective in treating extensive psoriasis, achieving relapse rates normally only seen with PUVA. TL-01 is easier to administer than PUVA, does not cause nausea and appears safe in pregnancy.

As shown by other workers using conventional UVB,[7] we have demonstrated that the addition of etretinate to TL-01 therapy enables a satisfactory result to be achieved with an approximate one-third reduction in the total exposure dose. The rapid relapse rate with re-TL-01 was disappointing but might well be improved by using TL-01 maintenance therapy.

In this study, re-PUVA appears an effective treatment for severe psoriasis, confirming previous findings.[8,9,17] As can be seen in Figure 1, the response to therapy was slower than in the TL-01 and re-TL-01 groups. This might be explained by the more rapid UV-dosage increments used in the latter (Table 1). As problems with etretinate toxicity were minimal, we consider that re-PUVA, by virtue of its dose-sparing effect, is indicated for patients potentially at risk of skin cancer due to either a high cumulative UVA dose (which we take arbitrarily at 750 J/cm^2) or the presence of other recognized risk factors.

The mechanism of the therapeutic effect of etretinate when combined with UVB or PUVA is not yet fully

Table 4. Summary of results

	Satisfactory response %	% in remission after 6/12 months	Comment
TL-01 ($n=12$)	80	50	Three patients withdrawn due to pruritus and burning
re-TL-01 ($n=14$)	93	33	One patient withdrawn due to pruritus and burning
re-PUVA ($n=15$)	100	50	One-third reduction in total exposure dose in comparison with TL-01

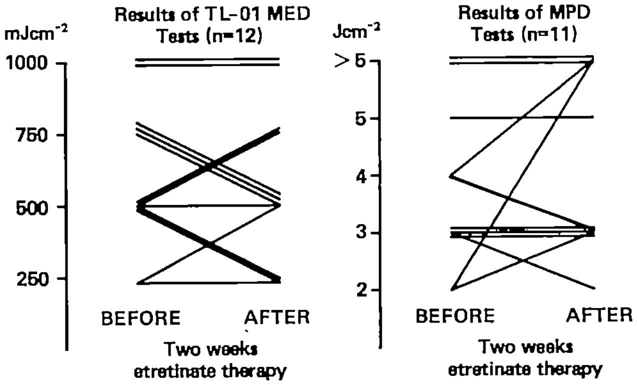

Figure 3. Results of MED and MPD testing before and after 2 weeks of etretinate therapy in the re-TL-01 and re-PUVA groups, respectively.

understood. Pretreatment with retinoids reduces both desquamation and infiltration of psoriatic plaques, raising the possibility of increased penetration of ultraviolet light.[16] Increased phototoxicity due to etretinate is an unlikely mechanism, as our MED and MPD comparisons before and after etretinate showed no significant change, supporting the work of Ippen who showed no significant difference in photosensitivity in 14 psoriatics before and after 3 weeks of etretinate.[18]

Among other polymorphonuclear leucocyte chemoattractants, leukotriene B$_4$ is present in increased amounts in lesional skin of psoriatic patients. Etretinate is known to inhibit the transepidermal accumulation of polymorphonuclear leucocytes in response to LTB$_4$.[19] It is of interest that *in vivo* and *in vitro* studies[20] have shown TL-01 therapy to inhibit the chemoattractant effect of LTB$_4$, and have also confirmed earlier reports of the efficacy of TL-01 therapy in this group of patients.[11,12]

This study confirms re-PUVA to be an effective therapy for severe psoriasis. The addition of etretinate to the TL-01 regimen decreases the number of treatments and the UV exposure dose required for clearing but does not extend the remission time as seen in re-PUVA or TL-01 therapy groups. In those patients for whom further PUVA is undesirable, TL-01 phototherapy, either alone or in combination with etretinate, may be considered an effective, although not necessarily safer, alternative.

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British Journal of Dermatology (1992) 127, 10–12.

Sun protection with hats

B.L.DIFFEY AND J.CHEESEMAN

Regional Medical Physics Department, Dryburn Hospital, Durham DH1 5TW, U.K.

Accepted for publication 4 February 1992

Summary

The degree of sun protection provided by various styles of hat at different anatomical sites on the head was measured using model headforms and ultraviolet-sensitive film badges. It was found that hats with a small brim, such as the flat cap favoured by elderly male photosensitive patients, provided negligible protection at all sites apart from the vertex and forehead. Peaked baseball-style caps offer good protection to the nose but are relatively ineffective at other sites on the face. Hats with a wide ($>7\cdot5$ cm) brim are necessary in order to provide reasonable protection factors (>3) around the nose and cheeks.

Measures which can be taken to protect the skin from excessive solar ultraviolet radiation (UVR) exposure are avoiding direct sunlight around the middle of the day, making use of shade, applying topical sunscreens and wearing suitable clothing, including hats.[1] In photosensitive patients, the face is commonly affected[2] and general advice on management often includes encouraging patients to wear a hat. Yet despite the conventional wisdom that wearing a hat is a useful photoprotective measure, there are few data on the quantitative benefits to be expected.

Previous studies[1,3,4] have shown that wearing a brimmed hat reduces exposure to the face, but little information has been provided with regard to protection at different sites on the head. The purpose of this study was to measure protection achieved at various anatomical sites on the head and neck from wearing different styles of hat. The sites chosen were the forehead, nose, cheeks, chin and back of neck because, in photosensitive patients, it is these areas of skin that are particularly affected.[2]

Methods

Ultraviolet radiation exposure was measured using the polymer film polysulphone.[5] The film readily degrades when exposed outdoors, and as a measure of the degree of solar UVR exposure the change in optical absorbance of the polysulphone film was measured at 330 nm. Badges were constructed by mounting the film in cardboard holders 30-mm square with a central aperture of 16×12 mm. These were attached to six sites (forehead, nose, both cheeks, chin and back of neck) on a PVC head form (model TH1170; Banbury Postiche Ltd, Banbury, U.K.) by means of 'Blu-Tack'. Six heads were mounted 45 cm apart along a rod 2·4 m in length. Different hats were placed on five of the heads and one head left uncovered. An additional film badge was placed on the vertex of the uncovered head. The assembly was placed outdoors and rotated at 0·4 rev./min for approximately 2–3 h during either a morning or afternoon (Fig. 1). Because the change of solar altitude was negligible during one revolution of the heads, their rotation at constant velocity may be regarded as equivalent to the random motion of a human subject outdoors. Measurements were made on six separate occasions on 20–23 August 1991 at Durham, England (latitude 55° N). During this period the weather was mainly sunny and

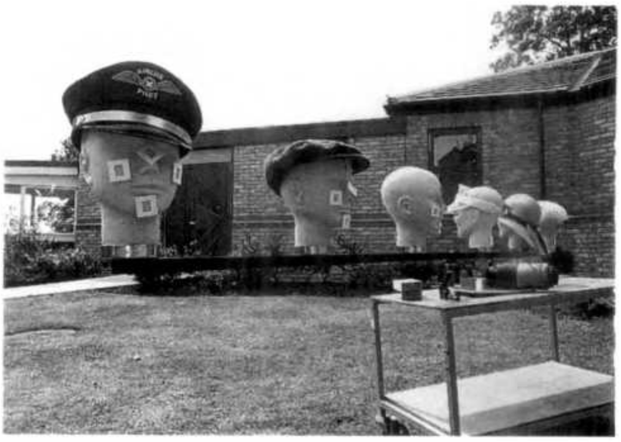

Figure 1. The heads rotating outdoors.

Correspondence: Dr B.L.Diffey

Table 1. Solar UVR exposure at various sites on head and neck

Hat position (Fig. 2)				Percentage of solar exposure relative to unprotected head					
Row	Column	Hat description	Hat classification*	Forehead	Nose	Left cheek	Right cheek	Chin	Back of neck
1	1	White sun-hat with large floppy brim	L	5	17	29	39	123	8
1	2	Brown tweed flat cap	S	7	70	79	107	140	105
1	3	Red cotton sun-hat	M	2	32	44	97	120	29
1	4	Straw boater	M	1	20	54	50	120	92
1	5	Blue hat with large floppy brim	L	2	9	10	13	41	5
1	6	Beige straw hat with large floppy brim	L	7	20	54	53	97	5
1	7	Brown corduroy flat cap	S	4	67	97	99	85	85
2	1	Green hat with large floppy brim	L	3	14	27	45	74	10
2	2	Airline pilot peaked cap	P	0	6	81	84	87	90
2	3	Green plastic visor (no crown)	P	6	8	45	49	72	94
2	4	Grey cotton baseball cap	P	1	65	114	106	92	72
2	5	Denim cotton peaked cap	P	3	45	91	82	96	89
2	6	Checked deerstalker cap	P	0	11	62	78	125	44
2	7	Checked flat cap	S	4	48	100	133	128	76
3	1	White cotton baseball cap	P	1	23	54	85	103	69
3	2	Red baseball cap with netting on crown	P	0	17	60	53	106	121
3	3	Trilby hat	M	3	69	88	90	94	75
3	4	Russian fur hat	S	24	93	105	132	109	50
3	5	Ladies pink hat	M	45	84	77	100	103	51
3	6	Tartan trilby hat	M	5	59	81	69	117	68
3	7	Straw sun-hat	L	6	28	64	67	126	56
4	1	Straw boater	L	38	68	86	83	102	73
4	2	Blue denim sun-hat	M	0	4	6	5	80	37
4	3	Brown trilby hat	M	24	81	108	124	109	66
4	4	Silk headscarf	—	77	95	115	126	97	0
4	5	White cotton cricketer's hat	L	4	2	16	0	78	16
4	6	Red cap with ear flaps	P	42	45	68	61	86	38
4	7	Straw sun-hat with large brim	L	4	7	44	32	63	93

*Hat classification: S, small brim (<2·5 cm); M, medium brim (2·5–7·5 cm); L, large brim (>7·5 cm); P, peaked cap.

the solar altitudes ranged from 31° to 47°. A total of 28 hats were used, including a ladies headscarf and a plastic sun visor with no crown (Fig. 2).

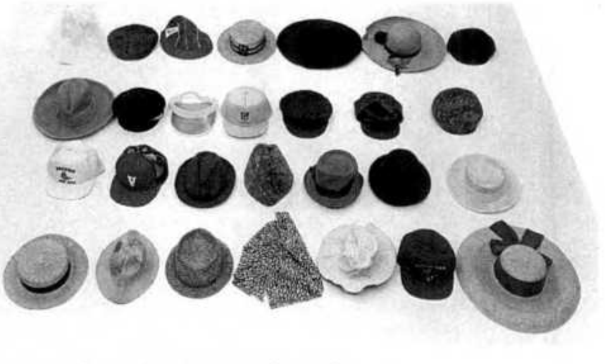

Figure 2. The 28 hats worn in the study.

Results

The ultraviolet exposure recorded by a given film badge was expressed as a percentage of that recorded at the same anatomical site on the uncovered head during the same period of exposure. This enabled an estimate to be made of the protection at a given site achieved by wearing a particular hat. These results are shown in Table 1. It can be seen that in some cases the percentage of solar UVR recorded at a site was higher than that received at the corresponding site on the uncovered head. We attribute this to differences in the orientation of the film badges at the same site between heads. By comparing the relative doses recorded on uncovered heads on the six separate occasions it was possible to estimate the uncertainty that differences in orientation were likely to contribute. These data are summarized in

Table 2. Solar UVR exposure on different sites of uncovered head relative to that recorded on vertex on each of six separate occasions

Site	Relative exposure on occasion 1	2	3	4	5	6	Mean ±1 SD
Vertex	100	100	100	100	100	100	100
Forehead	44	36	40	35	48	41	41±5
Nose	53	48	57	50	59	57	54±4
Left cheek	34	23	30	25	29	32	29±4
Right cheek	23	23	25	21	31	31	26±4
Chin	21	25	30	29	31	26	27±4
Back of neck	30	32	36	31	29	35	32±3

Table 3. Summary of sun-protection factors at various sites on the head and neck provided by different types of hat

Style of hat	Typical sun-protection factor: Forehead	Nose	Cheek	Chin	Back of neck
Small brim (<2·5 cm)	15	1·5	1	1	1
Medium brim (2·5–7·5 cm)	>20	3	2	1	2
Large brim (>7·5 cm)	>20	7	3	1·2	5
Peaked cap	>20	5	1·5	1	1

Table 2 and yield an overall coefficient of variation of 12%.

Discussion

The hats illustrated in Figure 2 can be classified into one of four categories: small brim (<2·5 cm); medium brim (2·5–7·5 cm); large brim (>7·5 cm); and peaked cap. The 'protection factor' provided at a given site by a particular style of hat is equal to the reciprocal of the fraction of UVR exposure recorded relative to the unprotected head. For hats in each of the four categories the data in Table 1 were reduced by calculating the geometric mean protection factor at each site to give the summary in Table 3. It may be seen that all types of headgear provide good protection to the forehead. Hats with little or no brim, as may be favoured by elderly male photosensitive patients, provide negligible protection at other sites on the head and neck. For people outdoors in sunny climates, such as Australia, a hat with at least a 7·5-cm brim is necessary to provide reasonable protection around the nose and cheeks, those sites on which non-melanoma skin cancers commonly occur.

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British Journal of Dermatology (1992) 127, 13–17.

Psoriasis: an audit of patients' views on the disease and its treatment

P.M.McHENRY AND V.R.DOHERTY
University Department of Dermatology, Western Infirmary, Glasgow G11 6NT, U K.

Accepted for publication 29 January 1992

Summary

This audit aimed to establish the effect of out-patient clinic attendance on patients' attitudes to their psoriasis, its severity and its treatment. A secondary aim was to determine if there was any correlation between the patients subjective concept of disease severity and objective medical assessment.

The audit was carried out by means of paired questionnaires completed by patients at their first visit to the out-patient clinic and at a review visit 3 months later. On each of these occasions an objective assessment of psoriasis severity score was made by the dermatologist.

We were able to confirm previous reports of major psychosocial effects of psoriasis on the life-style of many patients. At their first visit 67% (30/45) of patients stated that their life-style was affected by psoriasis, while at review 3 months later this figure was 40% (18/45). Overall 76% (34/45) of patients felt their psoriasis had improved following attendance at the clinic, while 11% (5/45) of patients felt it had deteriorated over the 3-month period. However, all patients stated they found attendance at the out-patient clinic to be of benefit. There was a significant correlation between the psoriasis severity score by the patient and by the dermatologist ($P<0{\cdot}001$).

Psoriasis affects at least 1% of the British population causing varying degrees of morbidity.[1] Patients with psoriasis are frequently referred to hospital out-patient departments for both diagnosis and management advice. Given the chronicity of the condition and the necessity for the patient's active participation in its treatment, successful management of psoriasis requires full patient co-operation. The majority of psoriatic patients can be managed by topical therapy such as tar, dithranol, or topical steroids.

Considerably more attention has been given to the dermatologists' views on psoriasis therapy than to those of the patients. Most published assessments of psoriasis treatments use doctor-based measures of disease extent and severity such as the PASI scoring system.[2] This system does not include a measure of the patients' views. In clinical practice it is well recognized that while some psoriatics will tolerate a moderate amount of visible disease, for others a totally clear skin is the treatment aim. Likewise some patients are able and willing to devote regular time to treating their psoriasis while others are unable to do this for social, financial or physical reasons.

A number of studies have examined the effects of psoriasis on the patient. Jobling[3] sent a questionnaire to 291 members of the Psoriasis Association and received 186 (64%) evaluable replies. The questionnaire covered history of psoriasis, personal and social information and experience of treatment. Difficulties were reported in establishing social contacts and relationships by 84% (156 patients). Thirty-four per cent (61 patients) said the 'worst thing' about psoriasis was irritation and soreness, while 13% (25 patients) found the unpleasantness of treatment the worst aspect of their psoriasis. Many patients had treated their skin daily for years to achieve and maintain an appearance satisfactory to them. There was dissatisfaction with treatment and also with doctor/patient relationships. At the time of the survey 79% of patients were regularly consulting their family doctor about their psoriasis and 56% were receiving treatment under the direction of a dermatologist. Stankler[4] used an interview technique to examine similar questions in a group of 100 psoriatic patients referred to a hospital out-patient department. It was again found that the 'worst thing' about having psoriasis was the embarrassment caused by its appearance and the restriction this placed on activities. Embarrassment was maximal when 'socially visible' areas of the skin were involved.

More recently, Finlay and Kelly[5] tried to construct a

Correspondence: Dr V.R.Doherty, Department of Dermatology, Victoria Hospital, Kirkcaldy, Fife, KY2 5AH Scotland, U.K

scoring system for psoriatic patients requiring in-patient therapy, which could be used as a repeatable measure and record of disability caused by psoriasis. This involved the use of a questionnaire completed by an interviewer at the time of admission and again 4 weeks after discharge. A series of questions on the effects of psoriasis on various aspects of the patients' life-style were answered using a seven-point linear-scale method. From an initial list of 28 questions, 10 questions were selected as being the most relevant. These included questions on the effect of psoriasis on employment, housework, gardening, clothing, bathing, cleaning, sport, smoking and drinking. These formed the basis of a 'psoriasis disability index' which was found to be a repeatable method of disease assessment. They found that in an individual patient the total area of involvement was not a reliable guide to subjective disability.

As the majority of psoriatic patients are managed as out-patients we wished to assess patients' attitudes to psoriasis and to its out-patient treatment. Given that their needs were likely to differ from those requiring in-patient therapy, we constructed our own questionnaires based on the clinical experience of all five consultants and five trainee dermatologists within the department. It was hoped that the questionnaires would allow us to determine the effects of attendance at the out-patient clinic on the patients' attitudes and ability to cope with psoriasis, and to identify areas in which we could provide a better service for the patients. We also wished to determine if there was any correlation between the assessment of disease severity by the patients and by the dermatologist.

Methods

Each of the 10 dermatologists in the department (consultants and trainees) recruited five patients for the study. These were new referrals to the dermatology out-patients of the Western Infirmary during the period March–December 1990. At the end of their initial clinic visit, and once a management plan had been established, patients were asked to participate in the study. All patients gave verbal consent. Two questionnaires were completed by each patient, one at the time of their first attendance and another after 3 months of treatment or at the time of skin clearance if earlier. An objective measurement of psoriasis area and severity was carried out by the same dermatologist at the time of completion of each of the two questionnaires. Psoriasis severity and extent at different body sites were graded on a scale of 0–4, with 0, no psoriasis and 4, very severe psoriasis. Nail and joint involvement were also graded in a similar fashion. Patients were excluded if immediate in-patient therapy was required, or if out-patient systemic treatment was needed. Topical therapy was not standardized as the aim was to treat each patient optimally according to the severity and distribution of their psoriasis. All dermatologists were therefore asked to manage the study patients using their usual methods of treatment, explanations of disease and therapy, and frequency of follow-up visits.

Questionnaires were completed by the patients at the end of their consultation, but in the absence of the doctor to avoid interviewer bias.

Questionnaire 1

This consisted of 12 main questions dealing with the effects of psoriasis on social, sporting, and working life, previous and present treatments and expectations of hospital treatment. Note was also made of age, sex, duration of disease and total duration of medical treatment. Most questions were answered by simple Yes or No options or by short inserted answers. A patient severity score was derived by asking the patient to score six factors about their psoriasis at the time of attendance on a 0–4 scale according to severity. These included (i) amount of body with psoriasis, the degree of (ii) itch, (iii) pain/discomfort, (iv) scaliness, (v) embarrassment, and (vi) 'stopping you doing what you want to do'.

Questionnaire 2

The second or review questionnaire consisted of 11 questions designed to establish whether any change in the patient's lifestyle had resulted from hospital management. Answer options such as 'got better', 'got worse' and 'stayed the same' were given where appropriate. Ample space was provided for other comments. A patient-derived severity score was also obtained at this time.

Statistical analysis

Stepwise linear regressions were used to determine which question(s) best predicted patient and doctor severity scores as well as the changes in each of these over the 3-month period. Paired sample *t*-tests and tests for zero calculation were used as appropriate to investigate the initial, review and change in the doctor and patient severity scores.

Results

Forty-five patients completed two fully evaluable questionnaires (F=21, M=24). The age range of the study group was 13–71 years (mean 33 years, median 30 years). The duration of disease at initial presentation was very variable, from 7 weeks to 40 years (mean 11·4 years, median 5 years). Over the 3-month period, most patients had attended the out-patient clinic on three occasions. All patients felt that attending the clinic had been of benefit.

It was stated by 67% (30 patients) at their first visit that their lifestyle was affected by psoriasis, while at review this figure was 40% (18 patients). In the majority of patients an improvement was noted in the effects of psoriasis on sports or hobbies, social life and time lost from education or occupation (Table 1). Although in the first questionnaire only 13 patients stated that their social life was affected, at review 14 patients had noted an improvement in their social life while one patient noted deterioration. It was felt by 27 patients (64%) that the most distressing aspect of their psoriasis had improved. A considerable number of the patients (25% at first visit and 37% at review) found symptoms of itch, irritation or scaliness to be the worst aspect of their psoriasis (Table 2). The 'worst site' varied greatly from patient to patient, and was not necessarily only exposed sites (Table 3).

Overall, 34 patients (76%) felt their psoriasis had improved and in approximately half of these (16 patients) it had cleared (Table 4). Five patients (11%), however, felt their psoriasis had deteriorated. Thirty-four patients (76%) stated that their psoriasis had cleared or improved on the initially designated worst site or most bothersome area after treatment, although in 20 patients (44%) the most troublesome site changed between the two visits.

With regard to patient knowledge and satisfaction, (Table 5) 24 patients (54%) felt their hopes had been fulfilled and more than half the patients stated that they understood their psoriasis better and found it easier to live with. However, 19 patients (45%) still had unanswered questions about their psoriasis. These included questions such as, 'Will my children get it?', 'Will it come back?', 'Will it spread?'.

Table 2. What is the 'worst thing' about your psoriasis?

Factor	First visit (%)	Review (%)
Cosmetic	12 (33)	9 (27)
Embarrassment	6 (17)	10 (30)
Symptoms (itch, irritation, scaling)	9 (25)	12 (37)
Effect on others	4 (11)	1 (3)
Treatment	3 (8)	1 (3)
Fear it will get worse	2 (6)	0
Total replies	36	33

Figures refer to numbers of patients replying 'yes' to questions. Figures in parentheses indicate percentage of total replies

Although in general the patients' severity scores were higher than those of the dermatologist (Table 6) there was a significant correlation between the scores at both the first visit and at 3 months ($r=0{\cdot}66$, $r=0{\cdot}50$, respectively, $P<0{\cdot}001$). There was also a significant correlation between the changes in the doctor and patients' scores over the 3-month period.

As completion of a lengthy and time consuming questionnaire by every out-patient is not practical, we were interested in identifying those questions which

Table 3. Is there one area where your psoriasis bothers you most?

Site	First visit (%)	Review (%)
Scalp/head	6 (13)	4 (16)
Face	11 (23)	6 (24)
Arms	8 (17)	0
Hands	5 (11)	2 (8)
Legs	9 (19)	4 (16)
Flexures	6 (13)	1 (4)
Trunk	2 (4)	8 (32)
Total replies	47	25

Figures in parentheses indicate percentage of total replies In the first questionnaire two patients stated two sites

Table 1. Effects on life-style. Figures refer to patients answering 'yes' to the questions

		Review		
Question	First visit (%)	Better	Worse	Same
Have you lost time from work/education because of your psoriasis?	8 (20)	3	2	3
Have you had to stop sports or hobbies?	23 (49)	10	12	1
Has your social life been limited by psoriasis?	13 (28)	14	1	10

Table 4. Psoriasis outcome

Question	Yes (%)	No (%)	No answer
Did your psoriasis disappear?	16 (36)	28 (64)	1
Did your psoriasis improve?	34 (76)	11 (24) (5 worsened)	0
Did it improve in the most bothersome area?	34 (79)	9 (21) (4 worsened)	2
Did the worst thing about your psoriasis improve?	27 (64)	15 (36) (6 worsened)	3

Table 5. Patient knowledge and satisfaction

Questions with regard to your psoriasis	Yes (%)	No (%)	No answer
Do you understand it better now?	28 (70)	12 (30)	5
Do you find it easier to live with now?	28 (70)	12 (30)	5
Have your hopes for hospital treatment been fulfilled?	24 (60)	16 (40)	5
Do you have any unanswered questions?	19 (45)	23 (55)	3

Table 6. Correlation of the most important questions with the mean psoriasis severity score. Figures in parentheses indicate standard deviations

Responses to questions			Mean psoriasis severity score	
Is your life affected?	Have you stopped sports/hobbies?	No. of patients	Patient	Doctor
Yes	Yes	22	15·0 (5·0)	9·3 (5·0)
Yes	No	9	12·0 (2·8)	7·9 (2·5)
No	No	16	8·9 (3·2)	5·3 (2·8)

There is a clear indication of increasing severity score if answers to both questions are yes and also if the first question alone is answered yes.

would best predict the severity score of both the patient and the dermatologist. Using stepwise linear logistic regression analysis to look for correlations between individual questions and doctor and patient derived scores, the two most useful questions were, 'Does the state of your psoriasis affect your life in any way?' and 'Have you had to stop your usual sports or hobbies because of your skin?' Responses to either of these questions were almost equally important but the combinations of answers to these questions were the best predictors of severity score, particularly the severity score of the patient, $P<0{\cdot}001$ (Table 6).

Discussion

No previous study has assessed the effect of attendance at out-patient clinics on patients' attitudes to their psoriasis or documented any resulting change in their lifestyle. In this study 67% of the patients found that their lives were adversely affected by psoriasis, when first seen at the out-patient clinic. After out-patient treatment the majority (76%) noted an improvement in their psoriasis and almost half (44%) were able to restart sports and hobbies. It was encouraging to note that 70% of patients were happier with their psoriasis after hospital attendance.

The detailed nature of the questionnaire meant that more information was available than is obtained in a normal consultation. We were surprised by the number of patients complaining of symptoms of psoriasis rather than its cosmetic effects. This is important, as it is often suggested that psoriasis is asymptomatic. It was also of interest to us that the 'worst site' was not always predictable and not always socially visible.

It appears that communication between doctor and patient, and dissemination of information concerning psoriasis could be improved, as a number of patients had unanswered questions. Written information sheets could improve this, but as the main source of patients' knowledge about psoriasis is from dermatologists,[6] repeated explanations and reassurance are necessary.

This study suggests that, in planning out-patient psoriasis management it is important to clearly document if, and how, the patient's lifestyle is affected by psoriasis, what the patient perceives as the most bothersome aspect of their psoriasis, and finally what are their hopes and expectations from attendance at the clinic. This knowledge would simplify formulation of a treatment plan with more appropriate individual goals. Patient-dictated goals could be used as outcome measures and/or as methods of assessing quality of care.

Acknowledgments

We would like to thank the following colleagues who assisted with this study, Professor R.M.MacKie, Drs W.N.Morley, J.N.S.Mitchell, R.S.Lever, D.M.Tillman, R.E.A.Williams, W.Perkins and G.I.Stables. We would also like to thank Mr T.Aitcheson (Department of Statistics, University of Glasgow) for his assistance with the statistical analysis of this paper.

We are grateful for financial assistance from the Greater Glasgow Health Board Audit Support Group.

Questionnaires available on request from Dr P.M. McHenry.

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British Journal of Dermatology (1992) 127, 18–21.

Smoking habits in psoriasis: a case control study

C.M.MILLS,* E.D.SRIVASTAVA,† I.M.HARVEY,‡ G.L.SWIFT,† R.G.NEWCOMBE,§
P.J.A.HOLT* AND J.RHODES‡

*Departments of Gastroenterology, *Dermatology, ‡Centre for Applied Public Health Medicine and §Medical Statistics, University Hospital of Wales, and University of Wales College of Medicine, Cardiff, U.K.*

Accepted for publication 19 February 1992

Summary

We have examined smoking habits in 108 patients with psoriasis, including some with palmoplantar distribution, and compared the results with matched controls from the community. There was a significant association between psoriasis, current smoking status (OR = 2·7, 95% CI 1·44–5·42, $P<0{\cdot}01$) and smoking habits prior to the onset of disease (OR = 3·75, 95% CI 1·68–9·47, $P<0{\cdot}001$). There was also a marked dose–response relationship; the relative risk of psoriasis in those currently smoking more than 20 cigarettes/day was significantly elevated (OR = 5·3, 95% CI 2·1–13·0, $P<0{\cdot}001$). Separate analysis of patients without palmoplantar distribution of psoriasis showed a significant association with smoking prior to onset of psoriasis (OR = 3·6, 95% CI 1·5–9·8, $P<0{\cdot}001$). Smoking may play a role in the aetiology of this common skin disorder.

Although there is little information available relating smoking to dermatological disorders, palmoplantar pustulosis has been shown to be associated with a high prevalence of smokers.[1] We have examined smoking habits in a large series of patients with psoriasis, including some with palmoplantar distribution, and compared the results with matched controls from the community.

Methods

One-hundred and fifty patients with psoriasis living in South Glamorgan were randomly selected from a diagnostic index in the department of dermatology. The index has been compiled over 20 years from all outpatient and in-patient records, without reference to severity of disease. For each patient, four randomly selected community controls were obtained from the Family Health Services Authority which holds a computerized register of patients from all family practices in South Glamorgan. Although a single control was obtained for each patient, four names were selected to give a number of substitute controls who might be used in the event of some failing to respond. We matched patients with controls for age (within 1 year), sex, and area of residence in an attempt to obtain pairs of similar social class. This was possible as the FHSA registers patients according to a locality code which is based on the electoral wards.

An initial calculation of sample size suggested that, on the basis of a paired analysis, 150 case control pairs would give 80% power to detect a 10% greater prevalence of smoking amongst cases above an assumed 25% prevalence amongst controls with a significance level of 5%.[2] The project was approved by the South Glamorgan Local Medical Ethics Committee.

A postal questionnaire was sent to each patient and to a randomly selected control from the four available. Patients who did not respond within 3 weeks were sent a reminder and were subsequently telephoned if necessary. One randomly selected control was contacted by post for each case. Where no response was received after one mailed reminder and a telephone reminder, a second randomly selected substitute was approached in the same way. A total of 159 controls were approached in order to obtain the 108 required (response rate 68%).

Data were obtained on current and previous smoking habits including the daily cigarette consumption and dates when patients commenced and discontinued smoking. The time of onset of their psoriasis and its distribution were noted, as well as observations about the effect of smoking and cessation of smoking on their skin disorder. Controls were asked similar questions about their smoking habits and whether they suffered from any skin complaint. Patients and controls were asked their occupation as an indicator of social class. Controls with psoriasis were excluded from the study

Correspondence: Professor J.Rhodes, Department of Gastroenterology, University Hospital of Wales, Heath Park, Cardiff CF4 4XW, U.K.

and a further control approached. Ex-smokers gave the date when they discontinued the habit.

McNemar's test of significance was used for paired data. Pairing was maintained in all analyses except for determination of the dose–response relationship. The results are expressed as the odds ratio; the P value is also given. The odds ratio (OR) is the measure of strength of association derived from a case control study which is numerically very similar to the relative risk of developing psoriasis in smokers compared with controls.

Results

One-hundred and eight of the 150 patients completed the questionnaire (72%); 56 were male. The mean age of the total group was 43·3 years (range 14–72). The median duration of the disease was 10 years (range 1–56 years). In 16 patients psoriasis involved the palmoplantar areas. Data on current smoking habits were incomplete for two pairs, and in 10 pairs there was incomplete data on smoking habits at the time of onset of psoriasis. A matched analysis showed a significant association between psoriasis and current smoking status (OR = 2·7, 95% CI 1·44–5·42, $P<0\cdot01$; Table 1). To examine whether smoking may be a consequence of psoriasis rather than a possible causative factor, an analysis of smoking habits immediately prior to the onset of psoriasis was performed. Each patient's smoking status immediately prior to the date of onset of psoriasis was compared with that of the matched control at the same point in time. This showed a significant association between smoking and the subsequent development of psoriasis (OR = 3·75, 95% CI 1·68–9·47, $P<0\cdot001$; Table 1). The overall percentages of current smokers among patients and controls were 46·2 and 23·6%, respectively; corresponding figures prior to the onset of psoriasis were 55·1 and 32·7%. A dose–response relationship was seen between the number of cigarettes smoked per day and the risk of developing psoriasis $\chi^2=13\cdot74$, $P=0\cdot002$; this was maximal in those currently smoking more than 20 cigarettes/day (OR = 5·3, 95% CI 2·1–13·0, $P<0\cdot001$). Unfortunately, the data available only permit this analysis for the current cigarette consumption. Information on cigarette consumption prior to the onset of psoriasis was not collected (Table 2).

Table 1. Current smoking status in 106 case control pairs; association between psoriasis and smoking (OR = 2·7, $P<0\cdot01$). Also smoking status prior to the onset of psoriasis in 98 case control pairs; association between smoking status at this time and psoriasis (OR = 3·75, $P<0\cdot001$)

	Cases	
	Smokers	Non-smokers
Current status		
Controls (smokers)	11	14
Controls (non-smokers)	38	43
Odds ratio = 2·71 (95% CL 1·44–5 42)		
Status at time of onset of psoriasis		
Controls (smokers)	24	8
Controls (non-smokers)	30	36
Odds ratio = 3·75 (95% CL 1·68–9·47)		

Table 2. Dose–response relationship between smoking and psoriasis in an unmatched analysis of 106 patients with psoriasis and community controls. Odds ratio (OR) and 95% confidence limits (CL) are given

Cigarettes smoked/day	Cases	Controls	OR	95% CL
0	57	81	1	—
1–9	9	5	2·6	(0·8–8·0)
10–19	14	13	1·5	(0·7–3·5)
20+	26	7	5·3	(2·1–13·0)

Analysis of patients with and without palmoplantar distribution of psoriasis (83 patients) also showed a significant association with smoking prior to the onset of psoriasis for the non-palmoplantar group (OR = 3·6, 95% CI 1·5–9·8, $P<0\cdot001$). Only 16 cases with palmoplantar pustulosis were identified in the group, and although there was an excess of smokers compared with matched controls (OR = 5·0, 95% CI 0·6–236, $P=0\cdot29$), the result was not statistically significant.

Analysis of the distribution of social classes for cases and controls showed no significant difference (χ^2 test $P=0\cdot33$) but the distribution was skewed towards the

Table 3. Social class composition of the cases and controls compared with the 1986 South Glamorgan intercensal survey. For simplicity they are grouped as non-manual (social classes 1, 2, 3 non-manual), manual (3 manual, 4, 5, 6) and economically inactive groups. A comparison of the proportions of cases and controls in non-manual and manual social classes shows no significant difference ($\chi^2=1\cdot87$ 1df $P=0\cdot17$)

	Cases (%)	Controls (%)	S. Glam (1986) (%)
Non-manual (1, 2, 3 NM)	56	35	25
Manual (3M, 4, 5, 6)	13	16	29
Economically inactive	31	49	46

upper non-manual groups compared with the 1986 South Glamorgan intercensal survey (Table 3).

Discussion

The results showed a striking association between smoking and psoriasis compared with matched controls, which remained significant when smoking status immediately prior to the onset of psoriasis was examined. There was also a marked dose–response relationship in the psoriasis group with the strongest association in those smoking more than 20 cigarettes/day ($OR = 5\cdot3$, 95% CI 2·1–13·0, $P < 0\cdot001$). All these relationships remained significant after exclusion of the group with palmoplantar pustulosis.

Confidence in the validity of these findings is enhanced by the fact that the original group was representative of both in-patients with severe disease and those regularly attending an out-patient department. The 72% response rate in completing the questionnaire is satisfactorily high. We accept the difficulty in avoiding recall bias as cases were aware of our interest in psoriasis and smoking habits, as several questions related to this, and were essential to obtain the information. Community controls are favoured as a means of overcoming some of the biases inherent in the use of hospital controls.[3] Attempts were made to minimize recall bias in controls by not revealing the suspected direction of the association. The prevalence of smoking in the control group is lower than that from the most recent General Household Survey (1988)[4] which identified 33% of adult males and 30% of females as smokers. Given this figure, we proceeded to examine the social class composition of our South Glamorgan patient and control groups by occupation. The patients and controls were reasonably matched but there was a tendency towards over-representation of the upper social classes compared with the 1986 intercensal survey, particularly in the patient group. Despite this, the patient group were heavier smokers than the controls of the general population. This might be expected to give smoking figures lower than the overall mean for the population. Furthermore, with present trends in smoking status, the true current values are probably lower than the 1988 figures by several per cent. The higher values for our subjects, which relate to the time of onset of their psoriasis 14 years ago (mean interval), are consistent with the steady decline in smoking habits during this period.

There is some literature on the relationship between psoriasis and smoking status. O'Doherty and McIntyre[1] studied the smoking habits of 216 patients with palmoplantar pustulosis and compared them with 626 hospital controls with miscellaneous dermatological disorders. At the time their disease developed 80% of the patients were smokers, compared with only 36% of the controls; four out of five patients were female. A more recent epidemiological study from Norway[5] identified 149 patients with psoriasis from a study population of 10,576. Amongst psoriatics daily smoking was more common (48%) than amongst non-psoriatic subjects (36%) with the greatest difference amongst females, although this was an unmatched analysis. Kavali *et al.*[6] in a large community survey of more than 14,000 subjects found 4·8% had psoriasis and this prevalence figure in males was greater in smokers compared with non-smokers; this difference, however, was inversely related to the number of cigarettes smoked, in contrast with our findings. Our figures confirm these findings in a mixed population with psoriasis, even after exclusion of the palmoplantar pustulosis group.

The smoking prevalence amonst patients may reflect the age, sex and social class of those patients seeking hospital attention for their skin condition. It has been found that increased alcohol consumption has been associated with psoriasis,[7] including a high consumption prior to the development of the disease; the same study failed to show an association between psoriasis and smoking. This study was restricted, however, to a population of young and middle-aged men. Previous epidemiological surveys have shown that alcohol consumption and smoking may be associated.[8] We did not gather information on alcohol ingestion in our case group, and accept that these two factors, smoking and alcohol, are related to each other. However, we feel the magnitude of the association and evidence provided by dose–response strongly suggest that smoking has a causative role rather than simply being an association. Lindelhof *et al.*[9] recently reported an unexplained increase in respiratory cancers in both male and female patients attending for PUVA therapy. They suggested that this may be related to the smoking habits of the group or perhaps a systemic carcinogenic effect of the PUVA treatment. Our study provides evidence that the smoking habits of the group may be at least partially responsible for this finding, as the majority receiving PUVA therapy are being treated for psoriasis.

There has been some speculation about the possible reason for an association between smoking and psoriasis. In patients with palomoplantar disease neutrophil leucocytes have shown abnormalities, but there was no significant difference in the morphological changes observed between smokers and non-smokers.[10]

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British Journal of Dermatology (1992) **127**, 22–25.

A prospective study into the value of patch and intradermal tests in identifying topical corticosteroid allergy

S.M.WILKINSON, A.H.M.HEAGERTY AND J.S.C.ENGLISH
Dermatology Department, North Staffordshire Hospital Centre, Stoke-on-Trent ST4 7PA, U.K.

Accepted for publication 7 February 1992

Summary We have prospectively performed patch and intradermal tests on 105 consecutive patients, attending for patch testing, to determine the optimum method of screening for corticosteroid hypersensitivity. Patch tests with Pivalone® and a corticosteroid series (all 1% in ethanol) detected all the patients with steroid sensitivity. However, intradermal tests were essential to exclude false positive reactions and detect all relevant steroid allergies in any individual patient.

Allergic contact dermatitis from topically applied corticosteroids is becoming increasingly recognized as a problem in the treatment of dermatoses.[1] In our department, allergy to topical hydrocortisone was found in 4·8% of 497 patients who were undergoing routine patch testing.[2] The steroid tixocortol pivalate, contained within the nasal preparation Pivalone®, has been shown to be a sensitive and specific patch-test marker of hydrocortisone sensitivity whilst hydrocortisone acetate in propylene glycol is not.[3] In patients who were patch-test positive to Pivalone®, an intradermal injection of hydrocortisone sodium phosphate was positive at 24–48 h in the presence of a negative hydrocortisone acetate patch test. Hydrocortisone-17-butyrate has also been suggested as a marker of corticosteroid sensitivity.[4,5]

In patients allergic to hydrocortisone, patch tests to steroids may be negative when intradermal tests with a pure sample of the steroid are positive at 24–48 h.[6] To establish the optimum method of screening for corticosteroid allergy, we have prospectively performed patch and intradermal tests on a series of patients attending for patch testing.

Patients and methods

All patients attending our contact dermatitis clinic were patch tested with hydrocortisone acetate (1% in ethanol), hydrocortisone butyrate (1% in ethanol), betamethasone valerate (1% in ethanol), clobetasol propionate (1% in ethanol), Pivalone® (as is), Preferid® ointment (as is) and Synalar® cream (as is) in addition to a standard battery of allergens. Patch tests were performed using Finn® chambers mounted on Scanpor® tape left *in situ* for 48 h. The patch tests were read at 48 and 96 h. Reactions were scored as recommended by the International Contact Dermatitis Research Group (ICDRG) and were considered positive when an indurated erythematous (+) reaction or greater was present. Intradermal tests were performed with 1 mg of hydrocortisone sodium phosphate in a volume of 0·1 ml (as Efcortesol®), hydrocortisone butyrate (pure steroid in 0·9% NaCl), betamethasone valerate (pure steroid in 0·9% NaCl), clobetasol propionate (pure steroid in 0·9% NaCl) and budesonide (pure steroid in 0·9% NaCl). Where a steroid was poorly soluble a suspension was used. The tests were read at 48 h and were considered positive if an area of indurated erythema >0·5 cm was present.

Results

One-hundred and five consecutive patients were studied and the positive results are summarized in Table 1. There were 11 patients (10·7%) who were positive on patch and intradermal testing to corticosteroids, nine to hydrocortisone (Cases 1–9), one to betamethasone valerate (Case 11) and one to hydrocortisone butyrate and budesonide (Case 10). Two patients were also positive on intradermal testing to other steroids despite a negative patch test (Cases 4 and 6).

Three patients were positive on patch testing but had negative intradermal tests. Case 12 was positive to Preferid® ointment on patch testing, with a positive reaction to 20% propylene glycol which is a constituent of the ointment base, but was negative to 2% propylene

Correspondence: Dr S.M.Wilkinson, The Skin Hospital, Chapel Street, Salford M60 9EP, U.K.

Table 1. Summary of positive patch and intradermal (*) test results

	Patient													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pivalone®	+	+	+	+	+	+	+	+	+					
1% hydrocortisone acetate			+					+					+	
1 mg hydrocortisone sodium phosphate*	+	+	+	+	+	+	+	+	+					
1% hydrocortisone butyrate										+				
1 mg hydrocortisone butyrate*				+		+				+				
1% betamethasone valerate											+			
1 mg betamethasone valerate*						+					+			
1% clobetasol propionate														+
1 mg clobetasol propionate*														
Preferid® ointment										+		+		
1 mg budesonide*										+				
Synalar® cream			+											+
Cetylsteryl alcohol												+		+
2% propylene glycol				+	+									
20% propylene glycol				+	+							+		

Table 2. Summary of clinical data in patients allergic to topical corticosteroids

Patient no.	Age	Sex	Site of dermatitis	Duration (years)	Personal atopy	Other positive patch-test results
1	55	F	Leg ulcer	25	–	Lanolin, neomycin
2	37	F	Hands and feet	1	–	Balsam of Peru, fragrance mix, Kathon CG®
3	54	F	Perianal	20	–	Balsam of Peru, fragrance mix, quinoline mix, neomycin, benzocaine, ethylene diamine
4	66	F	Leg ulcer	1·5	–	Eucerin
5	66	F	Hands	4	+	Neomycin, quinoline mix, lanolin, nickel, ethylene diamine, sorbitan sesquioleate
6	54	M	Feet	0·08	–	Formaldehyde, paraphenylene diamine
7	74	F	Leg ulcer	15	–	Lanolin, fragrance mix, Kathon CG®
8	70	F	Hands	12	–	Thiuram mix, formaldehyde, balsam of Peru, Kathon CG, bronopol
9	45	F	Hands	1	–	Balsam of Peru, formaldehyde
10	73	F	Leg ulcer	3	–	Fragrance mix, balsam of Peru, quinoline mix
11	43	M	Generalized	0·17	–	

glycol. Similarly, Case 14 was positive on patch testing to Synalar® cream and cetylsteryl alcohol contained in the cream base. Two patients were positive on patch testing to the pure steroid 1% in ethanol but not following intradermal injection (Cases 13 and 14).

The clinical features of the 11 steroid allergic patients are summarized in Table 2. The sample from which these patients were drawn comprised 36·0% men, 21·3% with a personal history of atopy, 41·7% with hand eczema and 10·7% with leg ulceration and stasis eczema.

Discussion

The prevalence of allergy to hydrocortisone in this sample at 8·7% is high. This may be explained, in part, by the large number of patients with leg ulceration

(10·7%) who in our district are commonly allergic to hydrocortisone.[7] In a previous study with a larger sample size[2] we found a frequency of 4·8%. Others[8] have noted tixocortol pivalate, and hence hydrocortisone, sensitivity to vary between 0·2% in Barcelona, and 3·4%, in Belfast.

In the past the significance of positive patch-test reactions to topical steroids has been uncertain. A use test in nine patients patch-test positive to hydrocortisone-17-butyrate was positive in only one when performed on normal skin.[9] However, a use test with hydrocortisone on eczematous skin was positive in nine of 11 of our patients who were patch-test positive to Pivalone® (unpublished observations). In addition, oral provocation with hydrocortisone, and systemic ACTH, have been shown to induce cutaneous reactions at the sites of allergic contact dermatitis caused by hydrocortisone and positive hydrocortisone patch tests.[10,11] Hydrocortisone has also been shown to stimulate lymphocyte proliferation in an *in vitro* test when Langerhans cells were used as the antigen-presenting cells.[12]

Pivalone® detected, in this group of patients, all who were positive on intradermal testing to hydrocortisone sodium phosphate, confirming our earlier findings.[2,3] However, hydrocortisone acetate, hydrocortisone-17-butyrate and betamethasone valerate patch tests, each 1% in ethanol, failed to detect the patients positive on intradermal testing. Hydrocortisone acetate patch tests detected two of nine allergic patients, hydrocortisone-17-butyrate one of three patients and betamethasone valerate one of two patients. This agrees with our previous study in which corticosteroid patch tests failed to detect all patients with positive intradermal reactions.[6] It has been shown that the chromatographic purity of several steroids in ethanol fell, after 1 month's storage in a refrigerator, to 95–75%.[13] It can be concluded, therefore, that intradermal tests are necessary to absolutely exclude the possibility of corticosteroid sensitivity. That contact hypersensitivity may exist despite the presence of a negative patch test has been shown in situations where the effects of allergen and irritant[14] or multiple allergens[15] summate. On a more practical level, patch tests did detect all the steroid-sensitive patients in this group and may therefore be suitable for screening a population, as long as intradermal tests are used to elucidate other relevant corticosteroid allergies. Whilst hydrocortisone, tixocortol pivalate and hydrocortisone butyrate have all been recommended for screening for corticosteroid hypersensitivity[5] these three are not sufficient to detect all cases. Case 11 was allergic to betamethasone valerate but was negative on patch testing to these three steroids. We would suggest that the composition of a steroid series, to be used to screen for corticosteroid allergy, should reflect the pattern of local steroid usage.

Patient 10 was positive on patch and intradermal testing to budesonide but denied use of the commercial preparation. This is, therefore, a true cross-reaction with hydrocortisone butyrate, to which he was also allergic, and would agree with the findings of others who suggest that the two may cross-react.[16]

Two patients (Cases 12 and 14) were positive on patch testing to a pure steroid, 1% in ethanol, but were negative on intradermal testing. We have assumed that these were irritant false positive reactions. In our previous study[3] using propylene glycol as the vehicle, in an attempt to increase the penetration of hydrocortisone acetate, there was an even higher incidence of false positive reactions. This emphasizes the importance of intradermal tests in excluding an irritant or allergic reaction to the vehicle used in patch testing with pure steroids.

Further misleading results occurred when commercial preparations of steroids were used to patch test patients. Case 12 was positive to Preferid® ointment but not to the intradermal steroid. The constituents of the ointment when patch tested separately, at recommended concentrations, were negative. However, when propylene glycol was patch tested at 20% a positive reaction was obtained. We have previously seen a similar patient positive to Preferid,® negative to 2% propylene glycol but positive to 20% (unpublished observations). Uncertainties about the correct concentration for patch testing with propylene glycol[17] would have resulted in this patient's allergy to propylene glycol remaining unsuspected had patch testing been limited to a 2% solution as recommended by the ICDRG.[18] A further patient (Case 14) was positive on patch testing to cetylsteryl alcohol contained in Synalar® cream.

In conclusion, patch testing with corticosteroids is an adequate screen for corticosteroid sensitivity. Ideally the vehicle used should be non-irritant and non-sensitizing to reduce the number of false positive reactions, and the corticosteroid molecule should be stable in solution. However, following a positive reaction, intradermal testing should be performed to confirm positive patch-test reactions and to detect sensitivity to other corticosteroids.

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British Journal of Dermatology (1992) 127, 26–29.

Basal cell carcinoma in young adults

N.H.COX

Department of Dermatology, Cumberland Infirmary, Carlisle CA2 7HY, U.K.

Accepted for publication 8 February 1992

Summary The epidemiology and outcome of basal cell carcinoma (BCC) in adults aged 15–34 years were examined. Northern Region Cancer Registry data from 1979 to 1989 revealed a crude annual incidence of 37/100,000 total population and the incidence was constant in all age-groups during this period; 150 patients (1·2%) were aged 15–34 years. There was a small excess of females in the young age-group.

The outcome of BCC in young adults was determined using records of all patients registered over 5 years ago and additional information from family doctors where required. Patients with BCC in naevus sebaceous or with syndromes of which BCCs are a feature were excluded. Median duration of BCC pre-diagnosis was 3 years. Of 39 patients with completed 5-year follow-up data, 21% had either incomplete excision requiring further early treatment ($n=4$), a later local recurrence ($n=2$), metastatic BCC ($n=1$) or a subsequent further primary tumour ($n=1$); an additional three patients had two primary tumours at initial presentation. Median hospital follow-up was 12 months but the apparent disease-free interval in patients with recurrent or new BCC was frequently over 2 years.

BCC in young adults may not be suspected because it is uncommon. However, the high frequency of local recurrence and of multiple primary tumours indicates that careful follow-up is prudent in young patients with this tumour.

Basal cell carcinoma (BCC) is the most common malignant skin neoplasm in the U.K. and one of the most common of all malignancies.[1–4] Although it occurs in younger individuals with a variety of inherited syndromes, especially xeroderma pigmentosum and naevoid basal cell carcinoma syndrome (NBCCS, Gorlin's syndrome) or may develop in congenital lesions such as naevus sebaceous, BCC is generally said to be uncommon before the age of 40 years.[4] The frequency and behaviour of BCC in young patients is poorly documented but clearly important. In particular, it is unclear whether diagnosis of BCC in young adults identifies a group at high risk of subsequent skin neoplasia. This study addresses some aspects of the epidemiology and outcome of sporadic BCC in young adults.

Methods

All registrations of basal cell carcinoma in the Northern Regional Cancer Registry from 1979 to 1989 were analysed. NBCCS is coded separately and was not included. Age-group at the time of diagnosis and year of diagnosis were recorded, to determine the incidence of BCC in the Northern Region (1989 mid-year population estimate 3,073,000) and to determine any change in registrations of BCC in any age-group over the 11-year period.

To investigate the frequency of second tumours in young adults (age 15–34 years) with BCC, and to determine the outcome of treatment of the initial tumour, further details were requested for all registrations from 1979 to 1985 as 5-year follow-up data was potentially available for this group. The Cancer Registry does not release information regarding individual patients without the permission of the consultant in charge of the case, and initial requests to include patient data in this study were made direct to the clinicians involved by the Cancer Registry. The clinicians included dermatologists, plastic and general surgeons, and radiotherapists. Where consent was given, hospital records were requested. If 5-year follow up data could not be obtained for these records, a request was made to the General Practitioner for further information.

Additional information analysed included the site of the BCC, age at presentation and duration of the lesion prior to diagnosis (if recorded), risk factors if recorded, and histopathological type of the BCC. In any case where records suggested clinical or histopathological reason to

Correspondence: Dr N.H.Cox.

doubt the diagnosis, or where there was a long duration of preceding lesion, the original histology specimens were examined to confirm the diagnosis and to exclude BCC within naevus sebaceous.

Results

Cancer Registry data (epidemiological study)

Over the 11 years studied, there were 150 registrations of BCC in young adults aged 15–34 years. These accounted for 1·2% of the total of 12,533 registrations (Fig. 1). The mean number of registrations of BCC annually was 13·6 in the young adults and 1139 at all ages (crude incidences per 100,000 population 0·44 and 37, respectively). There was no significant increase in the number of registrations over the 11-year period in any age group. The sex ratio (F:M) of patients with BCC registered over the 11-year period, after adjustment for the sex ratio in the total population of the region in each age group, was 1·09 for the young (<35 years) group and 0·93 for the older group (>35 years), suggesting a slight excess of female patients in the younger group.

Case record data

Ninety-two young adult patients were registered in the 7-year period 1979–85. Permission to study these individuals was given in 66 cases (72%), but 12 of these were not included in the study because either case records had been destroyed ($n=5$), age was incorrectly recorded ($n=1$), there was doubt about the validity of the diagnosis ($n=2$), or the BCC had arisen in a naevus sebaceous ($n=4$). The remaining 54 patients were analysed for details recorded at presentation, but adequate 5-year follow-up data was only available for 39 (72%) of these.

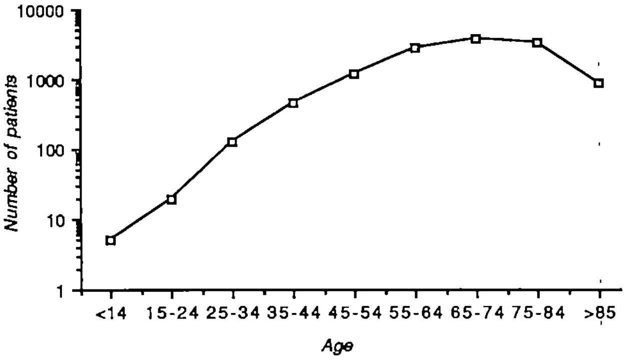

Figure 1. Registrations of basal cell carcinoma in the Northern region by age centiles (note logarithmic scale)

Table 1. Details of age, presentation, treatment, and follow-up in 54 young adults with BCC

Age	Range 15–34 years Median 30 years	
Duration of BCC before presentation	Range 3 months–>10 years Median 3 years	
Treatment	Surgery 45 Radiotherapy 5 Both 4	
Follow-up data:		
further surgery for incomplete excision		4
further surgery for local recurrence		2
metastatic BCC		1
second primary at presentation		3
second primary during follow-up		1

None of the case histories was suggestive of NBCCS and no patients had known carcinogen exposure or previous radiotherapy, but case records did not generally note any risk factors; only one patient was noted to sunburn easily.

Details of age at presentation, apparent duration of BCC before diagnosis, and treatment are shown in Table 1. A detailed description of site was recorded for 55 BCCs, of which 85% occurred on the head and neck (scalp 2, forehead 8, temple 2, cheek 7, eyelids/medial canthus 10, nose 13, lip 2); one was on the arm, and 10 (in seven patients) were on the trunk. Histology reports indicated a morphoeic pattern in 9%.

Follow-up data

Hospital follow up ranged from none to 10 years (median 12 months). At least 10% of patients defaulted from follow-up by 6 months.

Six BCCs were either definitely or possibly inadequately excised on histological grounds ($n=4$), or developed later local recurrence ($n=2$); both patients with a local recurrence, one of whom had multiple operations, had apparent disease-free intervals of 3 years during their follow-up. A further patient developed local lymphadenopathy and subsequent metastatic disease from a primary morphoeic BCC of the cheek.

One patient developed a second primary BCC 2 years after initial presentation (both were on the nose, but at different sites) and three patients had two trunk BCCs at the time of presentation, but no further tumours. A total of 11/39 patients (28%) with 5-year follow-up data therefore required further treatment for either potential or actual recurrence, for metastasis, or for second primary tumours. Three patients had further unrelated skin lesions excised (two naevi, one seborrhoeic keratosis).

Discussion

The biology of BCC has been reviewed in detail[1,2] but, apart from individual case reports,[5–7] there are few studies specific to this young age-group.[8–14] Some of these studies may be skewed by the inclusion of patients with tumours arising in congenital lesions (presumably organoid naevi) or with large numbers of BCCs (studies from surgical units have tended to exclude xeroderma pigmentosum but have not considered NBCCS).[10] A further doubt about the validity of some studies is the accuracy of histological diagnosis of BCC in this age group.[8,11] In the present study, the histology specimens were examined in any patient with a case history or a pathology report suggesting either diagnostic doubt, pre-existing lesions, or hair follicle differentiation in the BCC. The patients remaining in the study were those with apparently true sporadic BCC. The fact that at least 28% of patients in the present study had either recurrences or second primary tumours is further evidence that the index tumours were truly BCC, rather than trichoepitheliomas or other histopathologically similar but clinically benign tumours which occur in this age group.

The incidence of BCC varies considerably in different countries,[3,12,15,16] partly due to the effects of latitude. Studies in the U.K. rely on cancer registry data and may underestimate the frequency of skin tumours; a standardized incidence of 83/100,000 was recently reported in South Wales and noted to be much higher than the comparable registry data.[17] Although the present study also depends on registry data, the validity of the epidemiological results is supported by the fact that the proportion of patients aged under 35 years (1·2%), the site distribution (85% head and neck) and the more detailed sub-site distribution were all similar to results of other epidemiological studies.[10,12,15,17,18] Registrations of BCC were virtually constant in all ages over the 11 years of the present study, although a rising frequency of both squamous cell carcinoma[19,20] and BCC[19,21] (3% annual increase) has been demonstrated in the U.S.A. and interpreted as due to increased sun exposure.[21] The possibility that women develop BCC at an earlier age than men[22] is supported by a large Australian study which demonstrated an overall male : female ratio of 2 : 1 for all non-melanoma skin cancer but a lower ratio of 1·2 : 1 in patients aged below 40 years.[12] The results of the present study also support the idea that BCC in females is relatively more frequent at a younger age.

Although BCC is rarely a cause of great morbidity, follow-up in young patients is of importance as it may identify a group at risk of developing further BCCs subsequently. Despite exclusion of patients with syndromes associated with multiple BCCs, four of the 39 young adults with completed 5-year follow-up data in the present study had two primary BCCs at presentation, or within the follow-up period. A similar frequency (2/36) was reported by Rahbari and Mehregan[11] in their study of young patients with BCC, although the follow-up period was not stated. There are no comparable figures for an older age-group in the U.K., although the frequency of a second primary tumour in the present study is lower than the reported rate of about 30%[9,19,23–25] for all ages derived from countries with a much higher frequency of BCC than the U.K. Although the lifetime risk of further BCC in young patients is unknown, a recent study in Arizona has demonstrated that two thirds of patients (all ages) with two BCCs will develop a third BCC,[26] and counselling young patients regarding sun exposure would thus appear to be prudent.

The potentially long interval between treatment and recurrence of BCC is a further reason for follow-up of this tumour. Although all recurrent or second primary BCCs were apparent within the first 3 years of the 5-year follow-up period in the present study, the median duration of hospital follow-up was only a year and was notable for a high default rate. Recent studies demonstrate that only about two-thirds of BCCs which recur locally do so in the first 2 years[24,26] and almost a fifth of recurrences were more than 5 years after treatment in one study;[26] a longer period of formal follow-up is therefore advisable.

Factors which contribute to the risk of local recurrence of BCC include a morphoeic histology which, although not generally thought to be related to the patients' age, was reported in 9% of patients in the present study compared with the 2% expected overall frequency according to larger reviews.[2] An aggressive but clinically subtle growth pattern of BCC has recently been demonstrated to be especially likely in young patients,[14] particularly women; this, and cosmetic concerns, may lead to inadequate treatment in this age-group. Similarly, the proportion of BCCs which metastasize is very low (less than 0·1%), but this is relatively more likely in a younger age group.[27] The patient with metastatic disease in the present study had a morphoeic BCC on the cheek, incompletely excised at initial surgery, and treated with radiotherapy after a second local operation. Such tumours are difficult to treat adequately[28] and at high risk for metastasis.[27]

The follow-up data in the present study has two main shortcomings. The first is the relatively small proportion of cases where adequate follow-up data were available. This is a predictable difficulty in a retrospective study involving young adults treated up to 11 years ago, many of whom have married, changed address or changed family doctor in the intervening period. However, this is still a relatively large study of BCC in this age-group. A further shortcoming of the present study is that some of the follow-up data was obtained from non-specialist sources but, as all further tumours during the follow-up period were confirmed by histological examination, the non-specialist follow-up information could have under- but not overestimated the frequency of further tumours or recurrent disease. Although similar data for an older age-group were not examined, the object of the follow-up component of the study was to determine whether young adults with BCC have a significant rate of second tumours or locally recurrent disease, and the results clearly indicate that this is the case. The equivalent situation in older patients is irrelevant to the argument that BCC in young patients is a significant tumour requiring adequate follow-up.

This study has demonstrated a high frequency of second primary tumours in young patients with BCC, and a significant rate of local recurrence. This, together with the tendency for metastasis, albeit rare, to occur in younger patients, indicates that sporadic BCC in young patients identifies a group with significant risk of further tumours, in whom a prolonged period of specialist follow-up is desirable.

Acknowledgments

I am grateful to all consultants who allowed me to include details of their patients, to all general practitioners who supplied additional follow-up data, and to Dr Rebecca Wagstaff who prepared the figure. I am indebted to Sandra Mawdesley (Information Systems section) and the staff of the Cancer Registry of the Northern Regional Health Authority for their help with this study.

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Purpura fulminans due to protein S deficiency following chickenpox

W.G.PHILLIPS, J.R.MARSDEN AND F.G.HILL*

*Departments of Dermatology and *Haematology, Birmingham Children's Hospital, Ladywood Middleway, Birmingham B16 8ET, U.K.*

Accepted for publication 17 February 1992

Summary We report a case of purpura fulminans associated with protein S deficiency following chickenpox in an 8-year-old boy.

Case report

An 8-year-old boy was admitted to hospital 10 days after the onset of chickenpox. Three days prior to admission he rapidly developed painful haemorrhagic plaques on the skin. The lesions appeared over 24 h and then remained static. He had no other symptoms. There was no personal or family history of similar episodes. He had previously been in good health with no significant illness and was not taking any medication.

Examination showed soft tissue swellings, 15–20 cm in diameter, on the medial aspect of both thighs (Fig. 1) and similar lesions measuring 5 × 7 cm on the medial aspect of the left arm. Central black areas with geographical margins were surrounded by bright erythema. There were scattered 5-mm crusted papules over the trunk, face and limbs consistent with resolving varicella. He was apyrexial.

Investigations showed: haemoglobin 11·1 g/dl; white cell count $12\cdot1 \times 10^9$/l (56% neutrophils, 40% lymphocytes); platelets 212×10^9/l; film normal. Prothrombin time 20 s (control 15 s), partial thromboplastin time 38 s (control 32 s), plasminogen 83 mg/l (50–100 mg/l), fibrin degradation products $> 20 < 40$ mg/l (< 10 mg/l). Total protein S 28 U/dl (76–137), free protein S antigen 9 U/dl (71–141), protein C activity 61 U/dl (64–136), protein C antigen 52 U/dl (65–131). The following are clotting factors, usually reduced in consumption coagulopathy, which were normal: factor VII 0·76 U/ml (0·5–2 U/ml), factor IX 0·74 U/ml (0·5–2 U/ml), factor X 1·0 U/ml (0·5–2 U/ml), Von Willebrand factor antigen 1·6 U/ml (0·5–2 U/ml). Indices of renal and hepatic function were normal. Other hypercoagulable states were excluded by normal antithrombin III activity (102%; normal level 75–140%), and absent cryoglobulins, lupus anticoagulant, and antinuclear antibody. Both parents had normal levels of protein S.

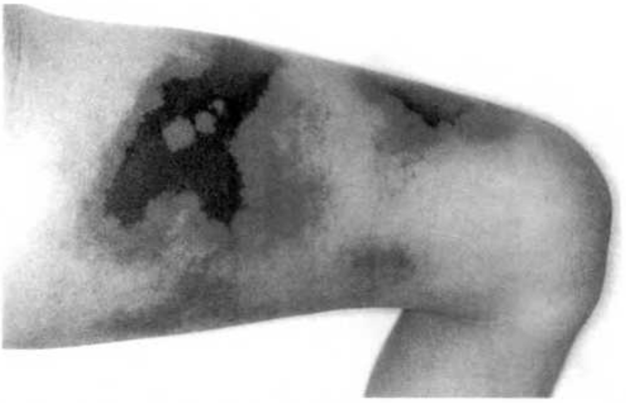

Figure 1. Purpuric lesions on the thigh with central necrosis and geographical margins, characteristic of purpura fulminans.

Skin biopsy from the edge of a lesion showed extensive epidermal and dermal necrosis, red cell extravasation into the dermis, and fibrin thrombi in capillaries (Fig. 2). There was no histological evidence of vasculitis.

He was treated with fresh frozen plasma and then anticoagulated with warfarin. There was no progression of the skin lesions following admission to hospital. The necrotic areas healed over a period of 8 weeks with severe scarring. There has been no recurrence after 18 months. When warfarin was stopped, free and total protein S concentrations returned to normal, at 80 U/dl and 93 U/dl, respectively. Protein C activity was also normal at 72 U/dl.

Correspondence: Dr J.R.Marsden.

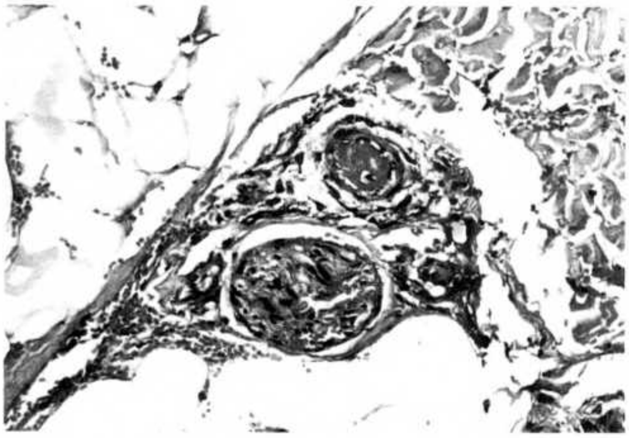

Figure 2. Photomicrograph showing a fibrin-filled vessel, and red cell extravasation into subcutaneous fat, reticular dermis and a cutaneous nerve (haematoxylin and eosin × 50).

Discussion

The differential diagnosis in this case included a septicaemic illness such as acute meningococcaemia, necrotizing vasculitis, and purpura fulminans. Acute meningococcaemia was excluded by the absence of systemic disturbance and negative blood cultures. Physical signs were not characteristic of vasculitis and there were no serological or histological features to support this diagnosis.

Purpura fulminans is a rare disorder seen predominantly following childhood infections such as meningococcaemia, chickenpox, rubella and scarlet fever. It is characterized by the sudden development of large areas of purpura with irregular geographical margins, associated with varying degrees of pyrexia, hypotension and consumption coagulopathy. The purpuric areas frequently undergo necrosis. Histological examination reveals thrombi in cutaneous capillaries and venules, associated with interstitial haemorrhage and varying degrees of cutaneous necrosis. Clinical, haematological and histological parameters all support the diagnosis of purpura fulminans in our patient.

Our patient had dramatically reduced levels of free and total protein S, associated with mildly raised fibrin degradation products, but no significant reduction of his platelet count or prolongation of prothrombin and partial thromboplastin times. Protein C activity and antigen concentration were below the lower limit of the normal range but were not sufficiently low to account for purpura fulminans. This suggests that protein S deficiency was the dominant abnormality, with intravascular coagulation clinically localized to cutaneous vessels occurring as secondary phenomenon.

Purpura fulminans can result from both inherited and acquired deficiencies of protein C and protein S. The normal levels of parental protein S, and previous report of purpura fulminans due to transient protein C and protein S deficiency following varicella[1] suggested an acquired deficiency, which was confirmed by the normal levels of protein S found after stopping warfarin.

Protein S and protein C are vitamin K-dependent proteins synthesized by the liver. When activated, protein C stimulates fibrinolysis and inactivates factors Va and VIIIa. Protein S is a cofactor for protein C.[2] Homozygous deficiency of protein C and homozygous deficiency of protein S have each been reported as causes of neonatal purpura fulminans.[3,4]

Acute deficiencies of protein C and protein S have been found in adults with meningococcal-induced purpura fulminans[5] and in an 18-year-old man with post-varicella purpura fulminans.[1] In these cases laboratory evidence of consumption coagulopathy was also present. It is not known whether low levels of protein C and protein S following such infections are of primary importance in inducing purpura fulminans or are secondary to consumption coagulopathy caused by other mechanisms.

Coumarin necrosis occurs shortly after starting anticoagulant therapy when protein C activity drops more rapidly than that of vitamin K-dependent clotting factors with longer half-lives. This is particularly likely to precipitate cutaneous necrosis in patients who already have low levels of protein C and protein S such as individuals with heterozygous protein C or protein S deficiency.[6,7] In the presence of documented protein C or protein S deficiency patients with purpura fulminans should be anticoagulated with heparin prior to the initiation of coumarin therapy. When purpura fulminans occurs in association with consumption coagulopathy fresh frozen plasma is the logical therapy, replacing both proteins C and S and clotting factors, and thus not putting the patient at increased risk of bleeding.

Systemic calciphylaxis is a syndrome occurring in patients with end-stage renal failure, characterized by soft tissue and vascular calcification, and cutaneous necrosis with clinical and histological features similar to purpura fulminans. Mehta *et al.*[8] have recently found reduced levels of protein C in patients with this condition compared with both normal controls and controls with end stage renal failure.

The clinical and histological similarity of post-infectious purpura fulminans to the skin lesions of homozygous protein C deficiency, homozygous protein S deficiency, coumarin necrosis, and systemic calciphylaxis is striking. The common finding of reduced levels of the physiological anticoagulants protein C and/or protein S in all these conditions suggests that deficiency of these proteins is a key factor in the pathophysiology of these disorders. We believe our case provides more evidence to support this hypothesis. It is not clear why the thrombotic complications of protein C and protein S deficiency particularly affect the cutaneous vasculature.

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Schöpf–Schulz–Passarge syndrome

B.E.MONK,* S.PIERIS† AND V.SONI†

*Departments of †Ophthalmology and *Dermatology, Bedford General Hospital, Kempston Road, Bedford MK42 9DJ, U.K.*

Accepted for publication 11 March 1992

Summary Three siblings, the children of a marriage between first cousins, are reported with the characteristic ophthalmological and cutaneous changes of the Schöpf–Schulz–Passarge syndrome. There have been only eight reported cases of this disorder, and it has not previously been described in Great Britain.

The Schöpf–Schulz–Passarge syndrome is a very rare form of congenital ectodermal dysplasia with a distinctive phenotypic expression. Only eight cases have previously been described.[1–5] The features are hypodontia, hypotrichosis, palmoplantar keratoderma, hypoplasia of the nails, and numerous cysts of the eyelid margins. There has also been a suggestion that there is an associated predisposition to skin cancer.[4] In this report we describe three siblings with the characteristic ocular and cutaneous changes. The family history in this case supports earlier suggestions of an autosomal recessive mode of inheritance.

Case reports

Case 1

A 62-year-old woman presented with a 15-year history of thickening of the skin of her palms and soles. Her parents had been first cousins, living in an isolated rural area of North Bedfordshire, U.K. She had a past history of a hypernephroma and of a basal cell carcinoma of the skin. She stated that her milk teeth had been commented upon as being abnormal at a school dental examination, and that no secondary teeth had ever erupted. An X-ray of her jaw, performed in her teens had shown no dental structures. On examination she was edentulous, had a curious bird-like facies (Fig. 1), and scanty scalp hair. Her palms and soles showed a diffuse keratoderma with a well-defined margin at the border between the dorsal and ventral surfaces. The fingernails were hypoplastic and longitudinally narrowed (Fig. 2), with an absent fingernail on the right little finger. The upper and lower eyelid margins of both eyes showed numerous 1–2-mm diameter cysts, some clear, and others showing a milky opacity (Fig. 3). Formal ophthalmological examination was otherwise normal. (This patient was presented to a clinical meeting of the Section of Dermatology of the Royal Society of Medicine, London, in October 1988.)

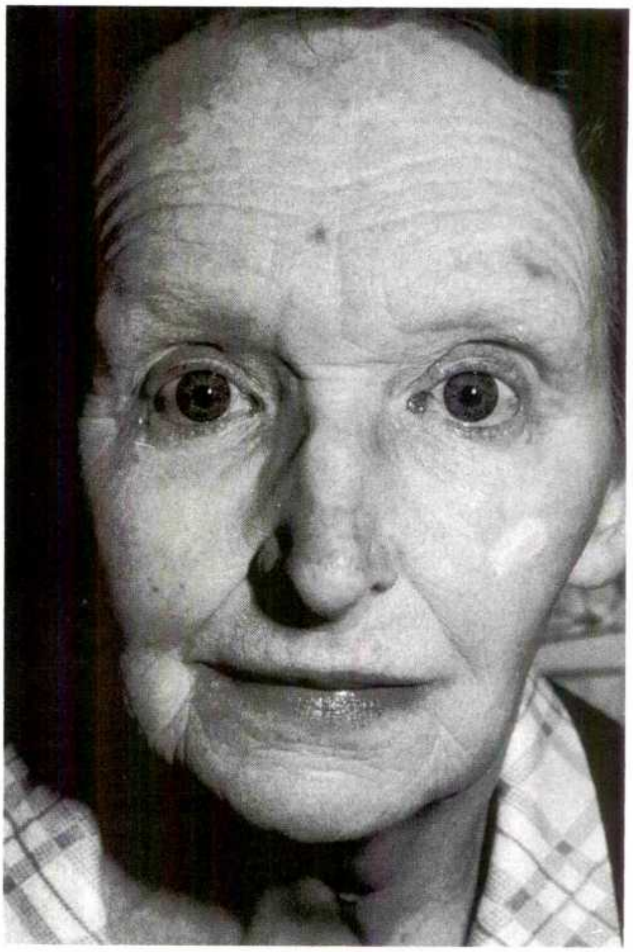

Figure 1. Case 1, characteristic facies.

Case 2

A 58-year-old brother of Case 1 presented with symptomless cystic lesions on the eyelid margins. He gave a

Correspondence: Dr B.E.Monk, Consultant Dermatologist, Bedford General Hospital, Kempston Road, Bedford MK42 9DJ, U.K.

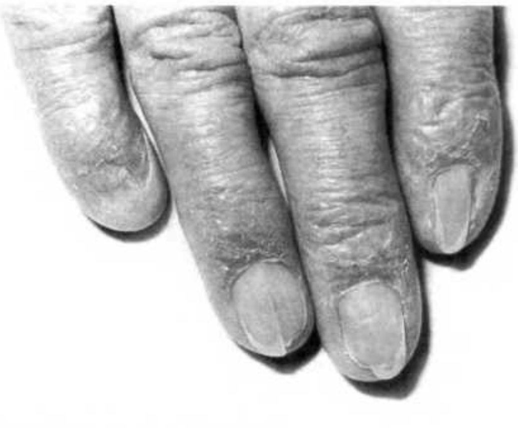

Figure 2. Case 1, hypoplastic nails.

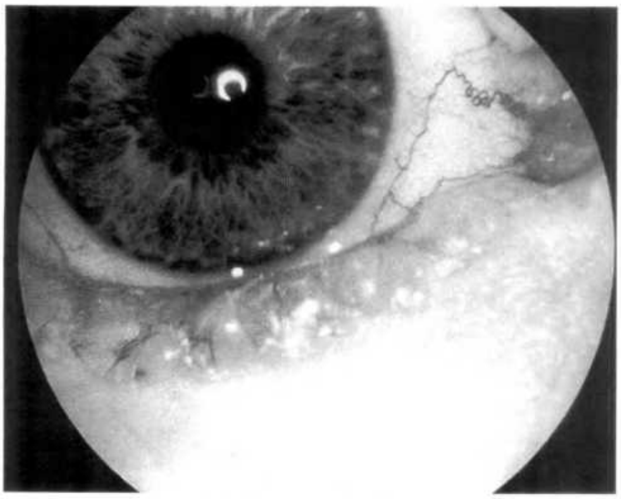

Figure 3. Case 1, translucent cysts on eyelid margins.

history of having had unusual primary dentition, and stated that no permanent teeth had ever developed. On examination he had a bird-like facies, strikingly similar to that of Case 1. He was edentulous, and had sparse hair (Fig. 4). There were numerous translucent and milky cysts along the eyelid margins, but no other ocular abnormality. The palms and soles were normal, and there was no abnormality of the nails. Histopathological examination of an excised cyst showed the features of a benign hidrocystoma.

Case 3

A 51-year-old brother of Cases 1 and 2, presented for review at our request. He had only had a single permanent tooth, which had subsequently been lost. His facial appearance was remarkably similar to that of his siblings, and his hair was very scanty, in a diffuse pattern. He was edentulous, and had a mild palmoplantar keratoderma, and thin, hypoplastic nails. There were numerous small cysts on the margins of his eyelids.

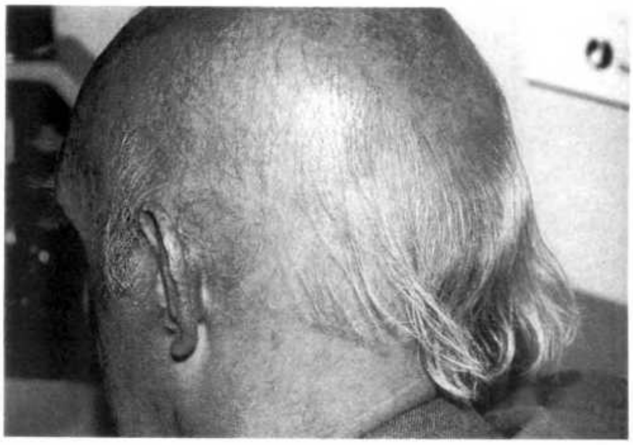

Figure 4. Case 2, demonstrating marked hypotrichosis.

The parents of these three siblings are said to have been unaffected, as are two other brothers. Cases 1 and 2 have no children, but Case 3 has an unaffected son. The two unaffected brothers both have normal children.

Discussion

The ectodermal dysplasias are a group of genetically determined disorders characterized by developmental abnormalities of the hair, nails, teeth, and sweat-duct apparatus. Well over 50 different syndromes have been described, distinguished by their pattern of clinical features and mode of inheritance, and these have been classified by Freire-Maia.[6] In 1971, Schöpf, Shulz and Passarge reported a previously undescribed form of ectodermal dysplasia, occurring in two elderly German sisters, born of a first-cousin marriage. These sisters had palmoplantar keratoderma, hypotrichosis, thin narrowed nails, hypodontia, and numerous small cysts along the eyelid margins. Three further, sporadic, cases were described by Burket *et al.*,[2] Font *et al.*[3] and Perret.[4] Two of these reports came from North America, and the third from the Netherlands. The patient described by Perret also had multiple squamous cell carcinomas of the skin. Nordin *et al.*[5] reported three elderly Swedish siblings who, in addition to the typical changes of the syndrome, all developed skin tumours. One had a basal cell carcinoma, and the others benign eccrine poromas.

The syndrome originally described by Schöpf, Schulz and Passarge appears to be a distinctive form of ectodermal dysplasia. Including the present report there have now been 11 cases described. The pattern of inheritance in this family would support the original contention of Schöpf *et al.* of an autosomal recessive disorder.[1] The syndrome has a number of unusual features. Firstly, ocular changes are not a typical feature of the ectodermal dysplasias, but the eyelid cysts seen in this syndrome appear to be distinctive and specific. The eyelid cysts should thus allow distinction from the Papillon–Lefèvre syndrome. Patients with the Schöpf–Schulz–Passarge syndrome may present to ophthalmologists who may not be particularly familiar with this group of disorders. The histological changes are those of a hidrocystoma, and presumably represent an anomalous development of the sweat ducts of the eyelids. None of the patients described has evidence of a generalized disorder of sweating, but in some forms of congenital ectodermal dysplasia hypohidrosis is an important manifestation. Secondly, patients seem to present in the second half of life, an age at which the possibility of a genetically determined disorder may not immediately be considered. Thirdly, five of the patients described (including Case 1 in this report) have had skin tumours, so that the possibility that this disorder represents a tumour-associated genetic syndrome must be considered, although in elderly, fair-skinned European subjects skin cancers are now so common that this finding may be merely fortuitous, and not an intrinsic feature of the disorder.

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British Journal of Dermatology (1992) **127**, 36–38.

Sweet's syndrome responding to cyclosporin

J.F.BOURKE, J.BERTH-JONES AND R.A.C.GRAHAM-BROWN
Department of Dermatology, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

Accepted for publication 28 February 1992

Summary We report a case of Sweet's syndrome which responded to treatment with cyclosporin 4 mg/kg/day. Cyclosporin is effective in the treatment of many dermatoses, and although the immunosuppressive effects of cyclosporin are mainly attributed to inhibition of T-lymphocyte proliferation, it has also been shown to affect the functions of both neutrophils and monocytes. Possible mechanisms of action of cyclosporin in Sweet's syndrome are discussed.

Acute febrile neutrophilic dermatosis, Sweet's syndrome, is a condition of unknown aetiology comprising fever, neutrophil leucocytosis and painful erythematous plaques predominantly affecting the limbs, face and neck. It is characterized histologically by a dense dermal polymorphonuclear leucocyte infiltrate.[1] The treatment of choice is systemic steroids, which produce rapid resolution in the majority of cases. In a small minority, long-term steroid therapy is required to control the disease. Dapsone, colchicine, indomethacin, clofazimine and potassium iodide have also been reported to be effective in the management of Sweet's syndrome.[1–5] To our knowledge, this is the first report of Sweet's syndrome treated with cyclosporin.

Case report

A 36-year-old female presented in 1986 with a 12-month history of recurrent tender lesions on her right arm. Examination revealed a large, tender, erythematous, indurated plaque on her right upper arm, measuring 8 cm in diameter (Fig. 1). She was apyrexial and otherwise healthy. There was no history suggestive of upper respiratory-tract infection. Full blood count, urea and electrolytes, liver function tests, and plasma protein electrophoresis were normal. Plasma viscosity was elevated at 1·81 cp (normal range = 1·5–1·72). Biopsy of one of the lesions revealed a dense polymorphonuclear leucocyte infiltrate in the upper dermis, oedema of the papillary dermis and prominent epidermal spongiosis (Fig. 2).

Repeated investigation over the 5-year period since her original presentation has revealed occasional leucocytosis (maximum WBC = $12{\cdot}3 \times 10^9$/l, neutrophils 90%) but no evidence of underlying malignancy.

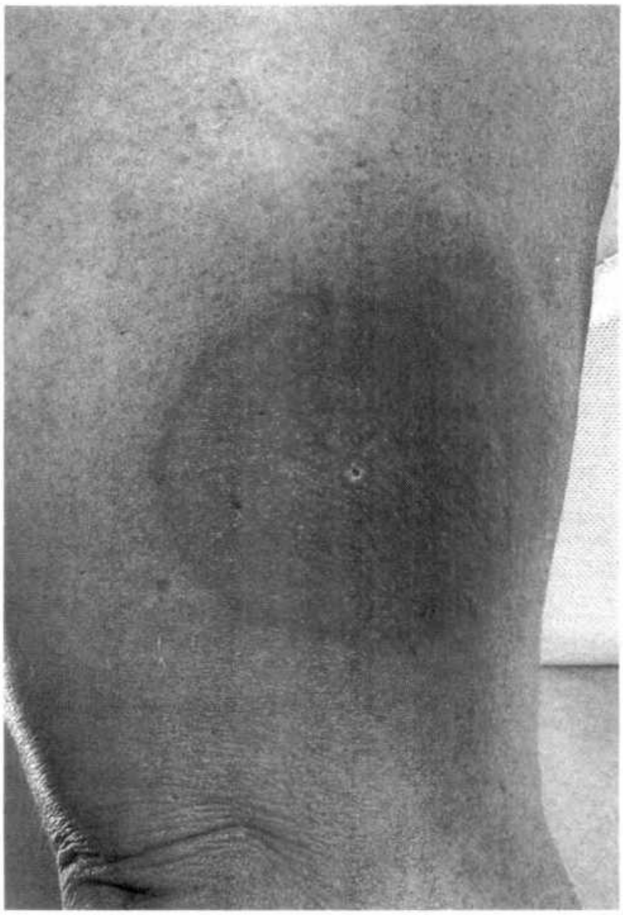

Figure 1. Tender erythematous plaque, 8-cm diameter, on the right arm.

The patient was started on prednisolone 30 mg/day, responded dramatically, and remained clear of disease for 18 months. Subsequently, however, she suffered frequent relapses. Ten such relapses occurred over the following $3\frac{1}{2}$ years, each lasting between 3 weeks and 4

Correspondence: Dr J.F.Bourke.

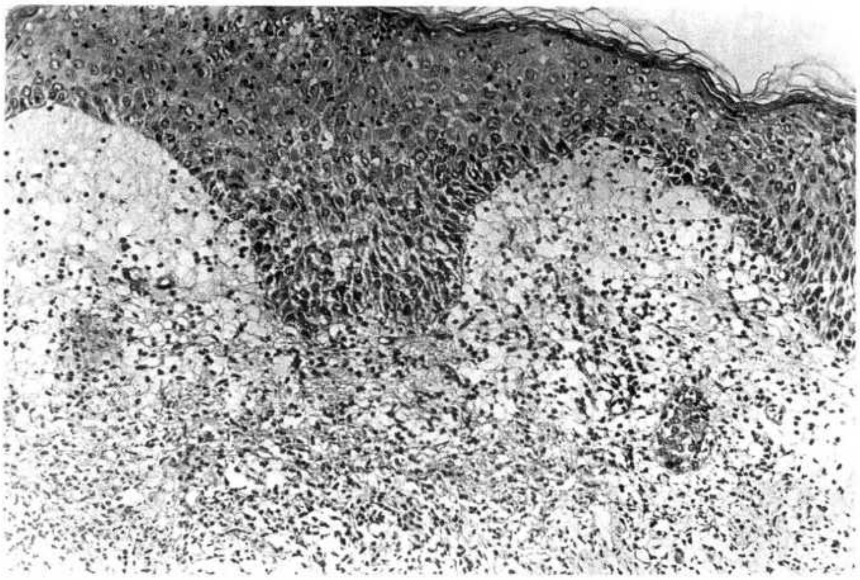

Figure 2. Skin biopsy showing a prominent neutrophil infiltrate and oedema of the papillary dermis (haematoxylin and eosin × 40).

months. On no occasion did her disease clear spontaneously.

A trial of dapsone 100 mg/day was partially successful but treatment was stopped because of persistent haemolysis. Sulphamethoxypyridazine 500 mg b.d. was substituted without benefit, and systemic steroids were again required to treat her residual lesions.

In August 1991 her condition relapsed and treatment with cyclosporin was started at a dose of 4 mg/kg/day. All lesions cleared in 5 days and the neutrophil count, which was slightly elevated prior to starting treatment ($8{\cdot}4 \times 10^9$/l), fell to within normal limits ($6{\cdot}4 \times 10^9$/l). Cyclosporin was continued for a total of 20 days. One week after stopping cyclosporin she developed a new lesion on her arm. Cyclosporin was recommenced at a dose of 2·5 mg/kg/day but failed to control the disease and, after 2 weeks, the dose was increased to 5 mg/kg/day. Complete resolution occurred within 7 days. To date, the patient has experienced no side-effects while receiving cyclosporin and there has been no consistent change in serum creatinine since starting treatment.

Discussion

This patient demonstrated characteristic clinical and histological features of Sweet's syndrome. Fever was not documented but is no longer considered an obligatory feature.[6] Control of her disease proved difficult. Continuous oral steroids, dapsone and sulphamethoxypyridazine were only partially successful.

In view of the refractory nature of this patient's disease, it was necessary to consider possible alternatives to repeated courses of corticosteroids. Cyclosporin was chosen because it is known to be effective in pyoderma gangrenosum,[7] a condition which has many features in common with Sweet's syndrome. Both conditions may present with acute tender papules, nodules or acneiform lesions which share the common histological feature of a neutrophil infiltrate, and both may be associated with pyrexia, arthralgia, autoimmune disorders and malignancy. These similarities, and the occasional co-existence of pyoderma gangrenosum and Sweet's syndrome[8] have led some authors to propose that they represent two ends of a nosological continuum.[9–12]

The immunosuppressive actions of cyclosporin are primarily mediated by an effect on T-lymphocyte proliferation. It does not initially appear to be a likely therapeutic agent in Sweet's syndrome, which is traditionally regarded as a neutrophil dermatosis. However, a number of recent reports have questioned the original concept of the composition of the dermal infiltrate in Sweet's syndrome. In one, a predominance of histiocytes was found in 11 out of 18 biopsies from patients with Sweet's syndrome.[13] In a larger study the predominant cell type varied with the age of the lesions. An initial preponderance of lymphocytes was followed by a predominance of neutrophils and then monocytes.[14] If an early predominance of lymphocytes does occur, this suggests a cellular immune mechanism and the effect of cyclosporin could then be explained on this basis alone.

However, cyclosporin is now known to have a much wider range of actions than initially outlined. In particular, it has recently been shown, *in vitro*, to inhibit neutrophil chemotaxis[15] and, more importantly, to impair neutrophil migration into infective and sterile inflammatory foci *in vivo*.[16] Monocyte functions are also modulated by cyclosporin and it has been shown, *in vitro*, to inhibit antigen presentation and suppress monocyte activation.[17,18]

Thus, the effectiveness of cyclosporin in Sweet's syndrome could be explained by an effect on lymphocytes, neutrophils or monocytes, or perhaps by inhibiting all three cell types.

Finally, it has recently been suggested that interleukin 1 (IL-1) might play a key role in Sweet's syndrome.[19] IL-1 possesses endogenous pyrogen activity, is chemotactic for neutrophils, induces a neutrophil leucocytosis and stimulates synthesis of prostaglandin E_2.[20] IL-1 could, therefore, account for many of the features of Sweet's syndrome. Cyclosporin may inhibit the release of IL-1.[21]

The response to cyclosporin in this case is important because this drug may offer a new and effective treatment for refractory cases of Sweet's syndrome. It may also support the view that cells other than neutrophils are involved in the pathogenesis of this condition.

Acknowledgment

We thank Sandoz Pharma Ltd who supplied the medication in this treatment.

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British Journal of Dermatology (1992) **127**, 39–40.

Cutaneous *Alternaria* infection treated with itraconazole

S.W.LANIGAN

Department of Dermatology, Bridgend General Hospital, Bridgend, Mid Glamorgan CF32 1JP, U.K.

Accepted for publication 24 February 1992

Summary A case of cutaneous *Alternaria* infection in an immunocompetent patient is reported which cleared with a 30-day course of itraconazole.

Infections of the skin with the soil saprophyte and plant pathogen *Alternaria* are rare.[1] Most cases occur in immunocompromised hosts,[1–6] although infections in immunocompetent individuals are recognized.[7–10] Treatment of these organisms with antifungal agents has met with varying success.

Case report

A 79-year-old female presented complaining of a swelling overlying her left knee for 2 years. It had initially appeared as a blister before crusting over, and was no longer growing. The lesion caused no symptoms and there was no preceding history of trauma, or inoculation. A course of cephradine had not altered the lesion. The patient was an otherwise sprightly, fit 79-year-old with no past medical history of note, and was not taking any medication.

On examination there was a circular lesion 1·5 cm in diameter in the midline just below the left knee (Fig. 1). The surface was erythematous and moist, and the lesion appeared granulomatous. There was a fine collarette of scale around the lesion. A clinical diagnosis of clear cell acanthoma was made and an incisional biopsy performed.

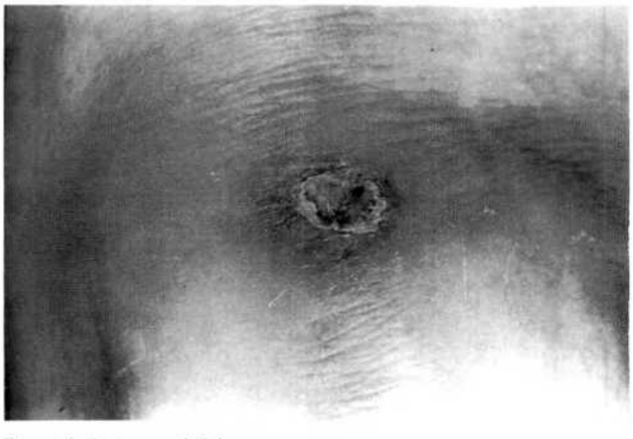

Figure 1. Lesion on left knee.

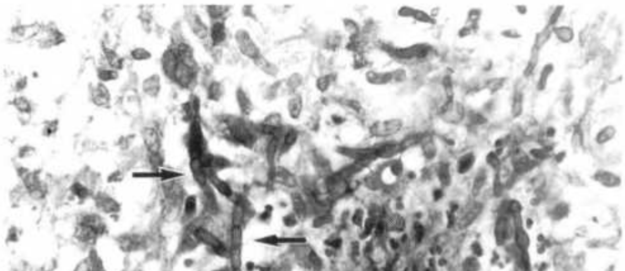

Figure 2. Photomicrograph showing inflammatory infiltrate, giant cells and fungal hyphae (PAS with haematoxylin × 330).

Histology revealed marked epidermal hyperplasia with underlying mixed chronic inflammation, including Langhans giant cells and necrotizing granulomas. Within the section was an infiltration with a bizarre hyphal fungus (Fig. 2).

Based on this histological appearance, a tentative diagnosis of a chromomycotic cutaneous infection was made and further material submitted to the Mycology Reference Laboratory at Colindale, London, for culture. An *Alternaria* species was isolated which could not be classified further. She was treated with itraconazole, 100 mg daily, for 30 days and there was some improvement in the lesion at the end of therapy. One month later, the lesion had healed completely, and 2 years later there is no evidence of recurrence.

Discussion

Alternaria is one of the genera of septate dematiaceous fungi which can cause phaeohyphomycosis. Other phaeohyphomycosis organisms include *Cladosporium*, *Exophiala* and *Curvularia*. *Alternaria* is a common fungus found in soil, but human infections are rare. Several cases have occurred in immunocompromised patients,[1-6] and in agricultural workers,[1,11] often after minor skin injury. The clinical features of cutaneous *Alternaria* infections are those of a focal eruption of reddish-brown papules and plaques which may ulcerate. Infection most commonly occurs on exposed skin or over bony prominences. Lesions develop gradually over a number of years.

Histopathological examination of cutaneous *Alternaria* infections reveals large, broad, septate hyphae extending from the stratum corneum downwards. Arthrospores may be readily seen. The inflammatory response is granulomatous, with giant cells and a neutrophilic infiltrate leading to microabscess formation.[12]

The organism is readily grown in culture and has a melanin-like pigment in its cell walls which gives the colonies a characteristic dark colour.

Treatment of phaeohyphomycotic infections has met with varying success, and a range of therapies has been advocated. These have included miconazole,[8,13] erythromycin,[8] amphotericin B,[5,19] and ketoconazole.[2,14] However, relapse after discontinuation of treatment and inconsistent results have led authors to recommend local excision if possible.[15,16]

Studies of the third generation triazole itraconazole have revealed *in vitro* activity against a variety of fungi including *Alternaria* species.[17] This drug has been shown to be an effective treatment in a small number of patients with *Alternaria* infections,[18,19] although relapse is still a problem.[19] However, in view of this drug's superior ease of administration and reduced toxicity compared with amphotericin B it was elected to treat this patient with itraconazole 100 mg daily for 30 days. A prompt response with complete healing of the lesion and no recurrence 2 years after treatment demonstrates the value of this drug in this rare cutaneous infection.

Acknowledgment

I would like to thank Professor Rod Hay for his help in diagnosis and management of this case, and Dr Alan Rees for preparation of the photomicrograph.

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British Journal of Dermatology (1992) **127**, 41–44.

Successful treatment of steatocystoma multiplex by simple surgery

M.KEEFE, BARBARA J.LEPPARD AND G.ROYLE
Royal South Hants Hospital, Graham Road, Southampton SO9 4PE, U.K.

Accepted for publication 18 February 1992

Summary

Steatocystoma multiplex is usually a minor cosmetic problem but rarely can be extremely disfiguring. Unfortunately, the very patients who would benefit most from treatment are usually regarded as having too may cysts for surgery. We report a case of extensive steatocystoma multiplex which was treated by a simple surgical technique under general anaesthetic.

Case report

The patient, a 51-year-old Malaysian male, developed skin cysts at the age of 7 years. The cysts gradually increased in size and number and became a major cosmetic problem. His parents and seven siblings were unaffected but one of his two sons had similar cysts. He first presented to one of the authors (B.J.L.) in 1975 when the cysts around the neck were the most cosmetically disfiguring (Fig. 1). These were punctured with a ball-tipped cautery point and the sacs extracted through small holes with a good cosmetic result (Fig. 2). He was not seen again until 1990 when he requested further treatment for cysts on the arms and scalp which had become more prominent. In the intervening years several cysts had been excised by a general surgeon, but the patient was disappointed with the resultant scars (Fig. 3).

There were about 300 large cysts concentrated over the flexor aspects of the forearms, behind the ears, around the neck, over the scapulae and over the central chest. Some clearly had a punctum and there were numerous associated comedones.

On this occasion cautery proved unsuccessful, because we had no artery forceps small enough to insert through the small puncture holes made by the ball-tipped cautery point to grasp the cyst lining. Puncture with a no. 15 blade, however, worked well. The cysts could not all be removed in this way because they were too numerous for individual infiltration with local anaesthetic. EMLA cream was tried but was unsatisfactory because, even though the skin itself was anaesthetized adequately for puncture, sac extraction remained very painful.

He was therefore given a general anaesthetic, and over a period of about 90 min nearly all the cysts on the arms and in the left post-auricular area were removed by all three authors working together (one on each arm and

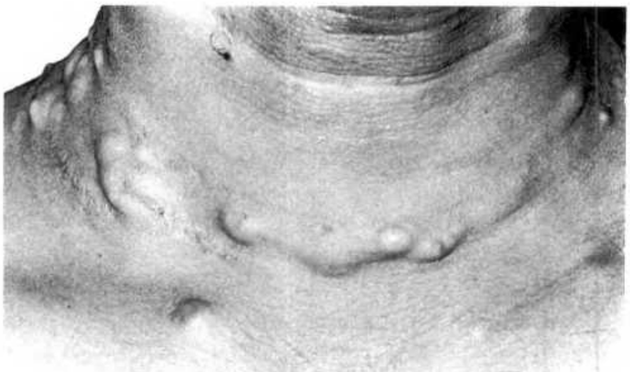

Figure 1. Prominent cysts on the neck in 1975, prior to treatment.

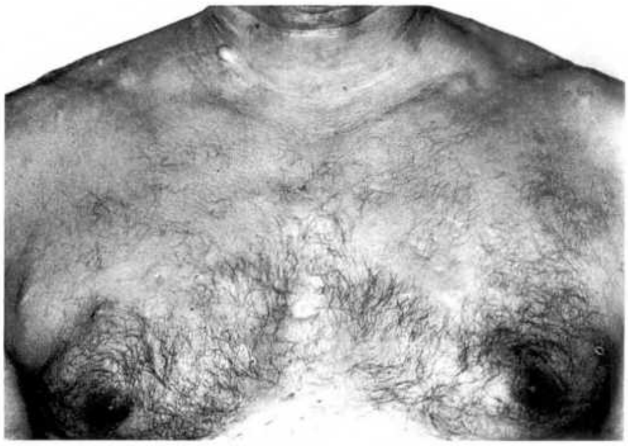

Figure 2. The neck in 1990 showing a good long-term result from cyst puncture and extraction.

Correspondence: Dr B.J.Leppard.

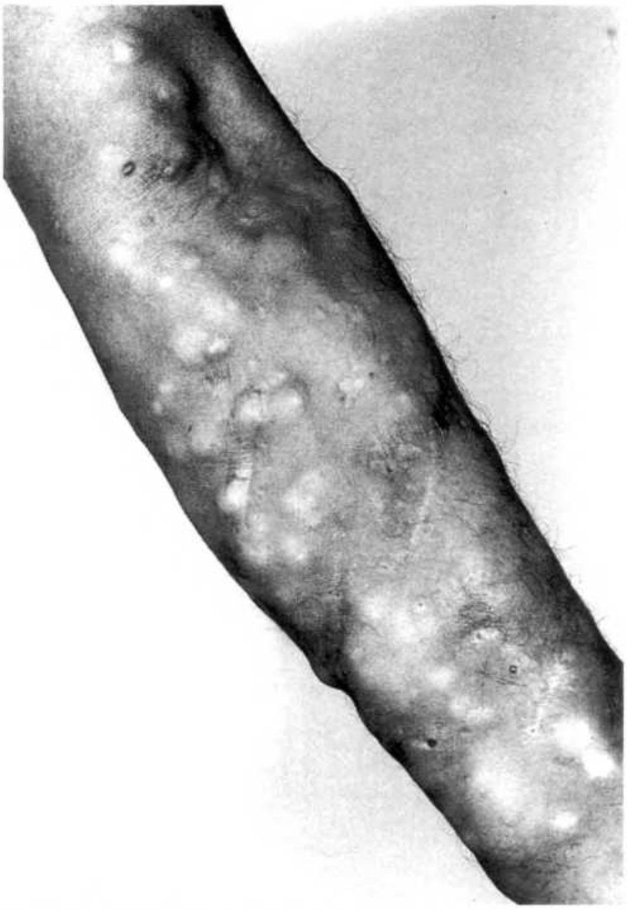

Figure 3. The left arm in 1990 showing the scars of previous cyst excisions.

the third dealing with the post-auricular area). The technique was to puncture each cyst with a blade, to squeeze out the contents, to insert a small pair of artery forceps through the puncture wound, grasp the base of the sac and pull it out. Often considerable force was needed to remove the complete lining of the cysts as they were adherent to the surrounding tissues; it is important to do so however, otherwise the cysts recur. Over 100 cysts were removed. The cysts on the right arm were removed through very small, 4-mm incisions, using a no.15 scalpel blade inserted at right-angles to the surface of the skin. The arm was wrapped in a sterile towel for 24 h post-operatively and healed uneventfully with a good cosmetic result. The scalp cysts were also removed through small incisions which were left open post-operatively and healed uneventfully. The author dealing with the cysts on the left arm preferred to make rather larger, 1-cm incisions and the arm was dressed with Melolin™ and a crepe bandage for 1 week. Despite prophylactic flucloxacillin, cellulitis developed in the left arm and some of the wounds took several weeks to heal. Three months later the cosmetic result was generally good, but better on the right arm than the left (Figs 4 and 5).

Several cysts were examined histologically and were typical of steatocystoma multiplex. There was a rather flattened contour, a thin undulating squamous epithelial lining and sebaceous glands within the cyst wall.

Discussion

Steatocystoma multiplex is an easily recognizable condition, characterized by multiple small cysts, and is inherited as an autosomal dominant trait. The cysts are skin coloured, yellow–white, or sometimes bluish in colour, averaging about 0·5 cm in diameter, and there are usually less than 20 in number. It is unusual for there to be huge numbers of cysts or for them to be larger than 1·5 cm in diameter. The distribution is usually characteristic but different in males and females. In males, cysts are found in a symmetrical distribution in a diamond-shaped area between the xiphisternum and the umbilicus, with some scattered over the back, particularly over the scapulae. In females, cysts are usually found in the axillae, the groins, the vulva and the centre of the chest.[1] This patient was unusual in the size, extent and distribution of his cysts but the histology was typical, and confirmed the diagnosis.

Cysts are usually noticed in the teens or early twenties and tend to appear almost simultaneously.[1] The rapidly developing cosmetic problem prompts patients to seek advice early in the course of the disease but, unfortunately, those with the most cysts are usually not considered suitable for treatment.

There are few reports of surgery for steatocystoma multiplex. In 1980 Holmes and Black reported successful surgical treatment of cysts of a size similar to those of our patient, but on the face.[2] In their patient an incision was made along the frontal hairline, the forehead skin was everted downwards to the eyebrows and the cysts removed from the underside of the flap. The flap was then replaced and sutured, giving a good cosmetic result. There is also a report of marsupialization of over 300 cysts in a single session by a plastic surgeon.[3]

Medical treatment has little to offer, although long-term, low-dose antibiotics may help prevent recurrent inflammatory lesions and isotretinoin has been reported as being useful when antibiotics have failed.[4,5]

Avakoff has reported a simple puncture technique for removal of 'sebaceous cysts'.[6] This involves puncturing the cysts with a scalpel, making an incision about $\frac{1}{4}''$

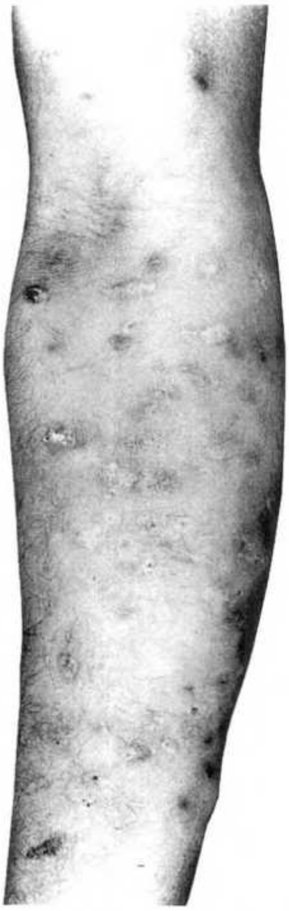

Figure 4. The right arm after multiple cyst extraction through small puncture wounds.

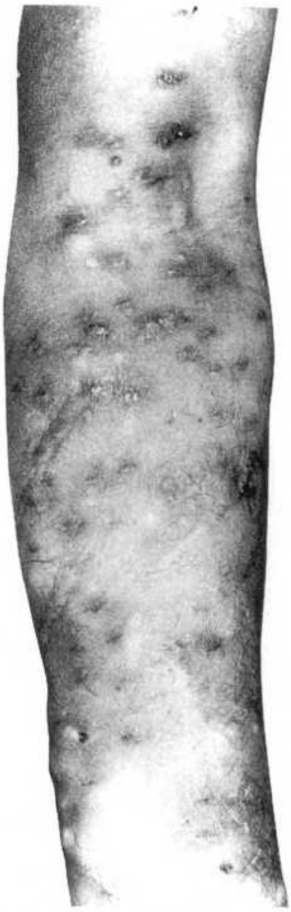

Figure 5. The left arm after multiple cyst extraction through larger incisions.

long; the contents are then squeezed out and the cyst lining removed with forceps. However, Avakoff did not specify which type of 'sebaceous cysts' were successfully removed this way. Indeed, the term 'sebaceous cyst' should be abandoned as the different types of cyst grouped under this heading have quite different clinical and pathological features. It is helpful to make the distinction before embarking on treatment. This technique is likely to work well for trichilemmal cysts,[7] but epidermoid cysts may be more difficult to remove as they are often adherent to the surrounding dermis. The reason for this is that bacteria from the skin surface get into the cyst through the punctum of an epidermoid cyst[8] and if pathogenic organisms rather than commensal organisms are involved the lining of the cyst wall is often replaced with granulation tissue and later scar tissue.[1]

We expected simple puncture and cyst extraction to be successful in this patient because few of the cysts had been inflamed. It proved to be possible to work through small, 4-mm incisions (the width of a no. 15 scalpel blade) and it appears important to do so, as the risk of infection is minimized and the resultant scarring is much reduced. We think general anaesthesia was justified because the patient is generally very satisfied with the results of treatment. His main complaint was that some of the cysts had been left. The small number of cysts which remained on the arms have now been removed under local anaesthetic. This procedure was time-consuming, but as new cysts do not appear after the first few years, surgery should give a good long-term result.

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British Journal of Dermatology (1992) 127, 45–48.

Minimizing the risk of post-operative pyoderma gangrenosum

C.C.LONG, J.JESSOP,* M.YOUNG† AND P.J.A.HOLT
*Departments of Dermatology, *Rheumatology and †Orthopaedic Surgery, University Hospital of Wales, Heath Park, Cardiff CF4 4XW, U.K.*

Accepted for publication 7 February 1992

Summary A 61-year-old woman with seropositive rheumatoid arthritis developed numerous ulcers due to pyoderma gangrenosum at suture entry/exit sites following an arthroplasty of the right hip when interrupted silk sutures were used to close the skin. When a subsequent arthroplasty was performed on the left hip and subcuticular Dexon sutures were used to close the skin only two small ulcers developed. Sixteen cases of pyoderma gangrenosum developing in surgical wounds have previously been reported.

We recommend that surgery in patients with a history of pyoderma gangrenosum is performed when the pyoderma is clinically quiescent, and that subcuticular sutures are used for skin closure, thus avoiding puncturing the skin surface.

Pyoderma gangrenosum (PG) may develop at sites of trauma or in surgical wounds.[1–14] We report a case of extensive PG developing in the surgical wound and at the suture entry/exit points following a right hip arthroplasty when interrupted silk sutures were used to close the skin. A subsequent arthroplasty of the left hip was followed by much less florid PG when subcuticular sutures were used to close the skin.

Case report

A 61-year-old woman with a 10-year history of seropositive rheumatoid arthritis was admitted to the University Hospital of Wales with increasing pain and stiffness of her hips. Radiographs showed marked degenerative changes in both hips, with bilateral protrusio acetabulae. She had a 7-year history of recurrent PG which usually developed on the dorsal aspects of both hands and on both knees.

In view of her increasing immobility bilateral hip arthroplasties were felt to be necessary. The risk of PG developing in the surgical wound post-operatively was considered, but because of her severe symptoms a Charnley total hip arthroplasty was performed on the worst affected (right) hip. Continuous Dexon sutures were used to close the muscle and fascia and interrupted silk sutures were used to close the skin.

Seventeen days post-operatively she was noted to have ulceration of the central portion of the wound site. Further ulcers appeared adjacent to the edges of the lower half of the wound. A striking feature was the involvement of the suture entry/exit sites (Fig. 1). The clinical appearance of the ulcers was typical of pyoderma gangrenosum. Culture of swabs taken from the wound produced a light growth of *Staphylococcus epidermidis* and diptheroids. The wound was cleaned with saline and dressed with povidone–iodine (Betadine®) ointment and a non-adherent gauze dressing. Her treatment with prednisolone was increased from 15 to 60 mg/day, and the PG began to heal. Her recovery was further complicated by a streptococcal septicaemia. The dose of prednisolone was gradually reduced and the PG eventually healed after 16 weeks.

Her other (left) hip became increasingly stiff and painful and 11 months after the first operation she was readmitted for an arthroplasty on this hip. Despite the complications that had followed the first operation she was most anxious to have the left hip operated upon.

A left Charnley arthroplasty was performed, and again the muscle and fascia were closed in layers with Dexon sutures. On this occasion a continuous subcuticular Dexon suture was used to close the skin, thus avoiding puncturing the skin surface. Steri-strips were placed across the wound.

Correspondence: Dr C.C.Long, Department of Dermatology (Outpatients Department), University Hospital of Wales, Heath Park, Cardiff CF4 4XW, U.K.

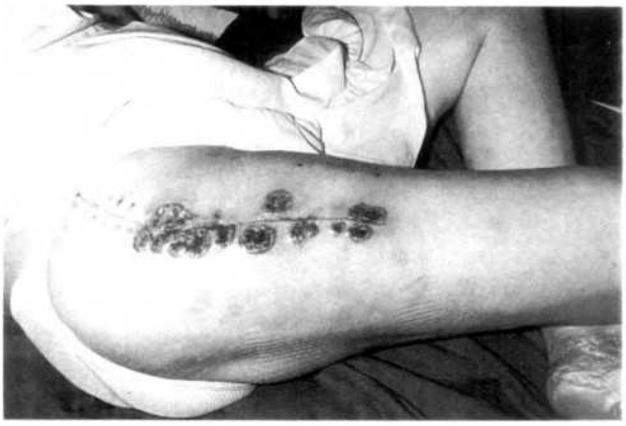

Figure 1. Multiple lesions of pyoderma gangrenosum arising at many suture entry/exit points following the first operation (photograph 19 days post-operation).

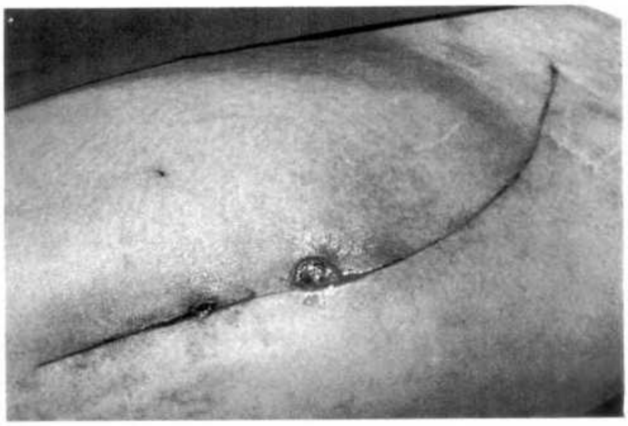

Figure 2. Two ulcers of pyoderma gangrenosum arising within the wound edges following the second operation (photograph taken 19 days post-operation).

Thirteen days post-operation she developed two small ulcers of PG within the wound (Fig. 2). However, the PG was much less florid than that following the first operation. The dose of prednisolone was increased from 10 to 60 mg/day, and then gradually reduced as healing took place over the following 3 months.

Discussion

Pyoderma gangrenosum is a painful, chronic, destructive, ulcerating, skin disease of unknown aetiology. It is often precipitated by trauma, and lesions most frequently occur on the legs.[1] In one series of 62 patients trauma was important in producing the ulcers in 20% of patients with ulcerative colitis, and 40% of patients with PG not associated with ulcerative colitis.[2] Powell *et al.*[1] reviewed 86 patients with PG, and found that the lesions were precipitated by trauma in 23 (27%). This phenomenon may be an example of pathergy similar to that seen in patients with Behçet's disease.[15]

PG has been reported as occurring in surgical scars, parastomal sites, skin grafts, intravenous catheter sites and in pleural aspiration sites, as well as at the site of injections, in patients both with and without an associated systemic disease.[3-14,16,17]

Sixteen cases of PG arising in surgical wounds have been reported (Table 1).[3-13] None of the reports specifies the methods of wound closure used, or mentions whether the PG developed at suture entry/exit points or in the wound edges alone. An associated systemic disease was present in seven of these cases. The PG developed within 1 week of the operation in nine of the 16 patients and within 2 weeks in a further two patients. Eight patients developed PG in other contemporaneous surgical wounds or puncture sites. Sternotomy scars following coronary artery bypass grafting operations accounted for three of the 16 cases, and in each case the vein donor site was also involved. Three of the patients gave a history of PG-like lesions developing in surgical wounds previously. Three cases of parastomal PG have been reported (Table 2).[14]

Shands *et al.*[11] reported PG occurring in the surgical scars of three members of one family (a woman, her mother, and her maternal aunt). One of these (the mother) had developed PG in each of three caesarean section scars. Two other family members also developed PG following trauma.

The ulcers healed in 15 of the 16 cases of PG developing in surgical wounds. Thirteen patients were treated with systemic steroids, and six healed within 2 months. In the one case where the ulcers failed to heal,[9] there had been considerable debate as to their aetiology. The ulcers had become extensive, and systemic steroids were only commenced after the disease had been present for 7 years.

The most striking feature of our case was the localization of the PG following the first operation, not only to the wound edges, but also to the suture entry/exit points. However, the whole wound was not involved, and neither were all of the suture sites. In the second operation a running subcuticular suture was used, together with Steri-strips, and although PG did develop, the lesions were much less florid. It is possible that puncturing both the epidermis and the dermis when inserting sutures increases the risk of PG developing within a wound.

Table 1. Cases of pyoderma gangrenosum in surgical wounds

Author	Patient age/sex	Operation	Underlying cause	Treatment	Outcome
Lamberty[3]	54/F	Strip varicose veins	None found	Systemic steroids	Healed
Lim, Bart[4]	63/F	Excision BCC (later grafted, PG recurred)	(Diabetes)	Topical and intralesional steroids	Healed
	48/M	Curettage and cautery of basal cell carcinomata	(Diabetes)	Intralesional steroids	Healed
Klevansky, Macrae[5]	43/F	PG grafted; PG developed at donor site	None found	Systemic and topical steroids	Healed
Beurey *et al.*[6]	68/F	Mastectomy (PG also developed on leg and buttock)	Monoclonal gammopathy	Systemic steroids, clofazimine	Healed
Schwaitzberg *et al.*[7]	35/M	Coronary artery bypass grafts, PG also developed at donor site and on back	Ulcerative colitis	Systemic steroids	Healed
	61/M*	Coronary artery bypass grafts, PG also developed at donor site	None found		
	68/M	Prostatectomy (Pfannenstiel incision)	(Ca prostate)		
Fulbright *et al.*[8]	61/M	Coronary artery bypass grafts	Not stated	Systemic steroids	Healed
Yaffee[9]	49/F†	Excision of cyst on ear, PG also developed at injection sites on buttocks	None found	Systemic steroids (7 years after onset)	Failed to heal
Carr *et al.*[10]	64/M‡	Cholecystectomy, PG also developed at venepuncture sites, and on right thigh and thumb	None found	Systemic steroids	Healed
Shands *et al.*[11]	28/F	Caesarean section	Not stated	Systemic steroids	Healed
	F (age not stated)	Caesarean sections × 3 (occurred after each operation)	Not stated	'Local measures'	Healed
	34/F	Tubal ligation (and appendicectomy)	Not stated	Debridement, systemic steroids	Healed
Cox *et al.*[12]	43/F	Strip varicose veins	Ulcerative colitis? (colectomy 10 years previously)	'Steroids'	Healed
Klein *et al.*[13]	16/F	Colectomy, PG also developed at intravenous catheter sites (ileostomy 10 weeks previously uncomplicated by PG)	Ulcerative colitis	Systemic steroids	Healed

*Past history of involvement of appendicectomy scar many years previously (1945).

†History of probable involvement of appendicectomy scar 9 years previously.

‡Underwent uncomplicated femoral angioplasty 6 months previously.

Silk sutures are known to cause a more marked dermal inflammatory response than synthetic sutures such as Dexon.[18,19] Within 1 week of insertion there is development of granulation tissue, with an infiltrate of histiocytes, fibroblasts, a few lymphocytes, and neutrophils. Later (at 2–4 weeks) the cellular infiltrate becomes less and the amount of fibrous tissue increases.[20] Possibly the use of interrupted silk sutures provided a focus of dermal inflammation from which the PG developed.

Table 2. Parastomal PG. All the patients had an ileostomy as a consequence of Crohn's disease

Author	Patient age/sex	Treatment	Outcome
McGarrity[14]	54/F	Systemic steroids	Healed
	29/F	Systemic steroids, azathioprine and revision of stoma	Healed
	30/F	(i) Systemic steroids and and relocation of stoma	Healed
		(ii) PG later recurred following revision/ relocation, treated with systemic steroids	Healed

The timing of surgery may also be important as a risk factor. In patients with ulcerative colitis, Crohn's disease, or rheumatoid arthritis there is no relationship between the activity of the disease and that of any associated PG. Possibly the activity of the PG itself is important. Before her first operation our patient had a lesion on her right knee which had healed only 1 week prior to surgery. In contrast, her most recent lesion prior to the second operation had healed some 8 weeks previously.

PG should be considered in patients whose wounds ulcerate or 'break down' post-operatively, particularly if they have a history of a similar episode in the past or of an associated systemic disease. Several factors may possibly influence the development of PG within surgical wounds. We would suggest that in order to minimize the possibility of PG developing post-operatively in patients who have a past history of the condition, that the operation is performed at a time when the PG is clinically quiescent. The wound edges should be closed with subcuticular sutures so that the skin surface is not punctured, and certainly the use of interrupted silk sutures should be avoided.

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Systemic and cutaneous plasmacytosis with multiple skin lesions and polyclonal hypergammaglobulinaemia: significant serum interleukin-6 levels

A.KODAMA, M.TANI, K.HORI,* T.TOZUKA,† T.MATSUI,† M.ITO,† S.NAKAO,† T.FUJITA† AND H.KOBAYASHI‡

Department of Dermatology, Kobe University School of Medicine, 5 Kusunoki-cho, 7 chome, Chuo-ku, Kobe 650, Japan
**Department of Dermatology, Kobe National Hospital, 1 Nishiochiai, 3 chome, Suma-ku, Kobe 654, Japan*
†Department of Internal Medicine, Kobe University School of Medicine, Japan
‡Kobayashi Dermatology Clinic, Suma-ku, Kobe 654, Japan

Accepted for publication 12 February 1992

Summary We report two patients who developed benign plasmacytosis with multiple skin lesions. The cases were characterized by hyperplasia of mature plasma cells, and polyclonal hypergammaglobulinaemia. One patient had hyperplasia of mature plasma cells not only in the skin, but also extensively in lymph nodes and the retroperitoneal areas around the ureters. The other had plasma cell hyperplasia limited to the skin. Extensive investigations failed to reveal any clinical or laboratory evidence suggesting the presence of any underlying disease accompanying the hypergammaglobulinaemia and/or plasma cell proliferation, such as chronic infectious disease, collagen disease or other chronic inflammatory disorder. Clinically and histologically, the first patient showed features compatible with a diagnosis of systemic plasmacytosis and the second with a diagnosis of cutaneous plasmacytosis. Significant serum interleukin-6 (IL-6) levels were detected in both patients, suggesting that IL-6 may be involved in the pathogenesis of these conditions.

Benign plasma cell proliferation with polyclonal hypergammaglobulinaemia may occur in the absence of underlying diseases such as chronic infectious disease, collagen disease, or other chronic inflammatory disorders. Plasma cells infiltrate and proliferate in the skin, lymph nodes and other organs to produce changes which include multiple skin lesions, and generalized lymphadenopathy. The skin lesions, characterized histologically by prominent hyperplasia of mature plasma cells, were first described in the English-language literature in 1986 when Watanabe *et al.*[1] reported two patients with peculiar multiple skin lesions, generalized lymphadenopathy and polyclonal hypergammaglobulinaemia, which they called systemic plasmacytosis. Similar cases had already been described in the Japanese-language literature.[2,3] Cases with cutaneous plasmacytosis alone[4,5] have also been reported. In these cases hyperplasia of mature plasma cells was restricted to the skin and presented as multiple cutaneous nodules or plaques accompanied by polyclonal hypergammaglobulinaemia. Lymphadenopathy was not present but the clinical and histological features of the skin lesions were similar to those of systemic plasmacytosis.

We report two patients whose clinical and histological features are compatible with the diagnoses of systemic and cutaneous plasmacytosis, respectively. In both cases an increase in serum levels of interleukin-6 (IL-6) was noted. Because IL-6 is a cytokine inducing the final differentiation of activated B cells into immunoglobulin-producing cells, these findings suggest that IL-6 may play a role in the pathogenesis of this entity.

Case reports

Case 1

A 54-year-old man was seen in June 1989 for evaluation of weight loss, fatigue and a high fever. These symptoms had been present since April of the same year. He had been treated with several drugs including antibiotics, antituberculous therapy, and systemic corticosteroids without improvement.

Physical examination showed multiple, well-demarcated, reddish-brown, rubbery hard nodules and

Correspondence. Dr M.Tani.

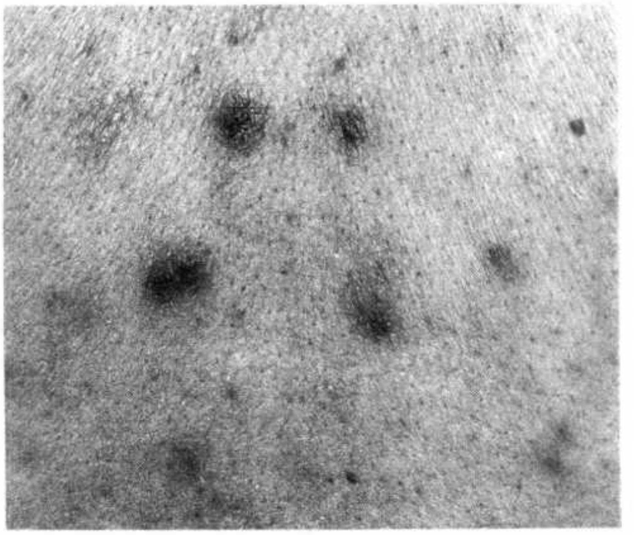

Figure 1. Case 1. Multiple reddish-brown nodules on the back.

plaques, measuring 0·5–3·0 cm in diameter, scattered over the trunk and nape of the neck (Fig. 1). There was bilateral asymptomatic enlargement of superficial lymph nodes in cervical, supraclavicular, axillary and inguinal areas. The liver and spleen were not palpable. Laboratory investigations revealed an elevated erythrocyte sedimentation rate (ESR, 177 mm/h), leucocytosis (11,300/mm^3) with 54·9% segmented neutrophils, anaemia (haemoglobin 5·2 g/dl), hypoalbuminaemia (1·3 g/dl) and elevated γ-globulin levels (5·71 g/dl). Serum immunoelectrophoresis showed polyclonal hypergammaglobulinaemia with IgA of 1193 mg/dl (normal 136–328 mg/dl); IgG, 6830 mg/dl (normal 940–2125 mg/dl); IgM, 614 mg/dl (normal 78–170 mg/dl). Renal function tests disclosed glomerular and tubular dysfunction with severe proteinuria and haematuria. Repeated examinations failed to detect Bence Jones protein in the urine. Additional laboratory values were as follows: C-reactive protein, positive; antistreptolysin O antibody, positive; serological tests for syphilis, negative; latex agglutination test, negative; and antinuclear factor, positive at a titre of 1:160. Computed tomograms, whole-body magnetic resonance imaging and ultrasonograms of the abdomen disclosed enlargement of multiple peripheral lymph nodes, mediastinal nodal involvement, hydronephrosis, thickening of both ureters, and multiple masses around the ureters. A skeletal survey was normal. Examination of bone marrow aspirates revealed a slightly hypercellular marrow with 82% granulocytes and 7·6% plasma cells. The plasma cells displayed no atypia.

Histology of a biopsy specimen taken from a plaque on the trunk disclosed a dense perivascular and periadnexal infiltrate in the dermis, which consisted largely of plasma cells with an admixture of a few lymphocytes and histiocytes (Figs 2 and 3). A biopsy specimen from a left cervical lymph node revealed proliferation of mature plasma cells in the medulla and interfollicular areas without destruction of the lymph node architecture. Biopsy specimens obtained from the left periureteric fibrous masses showed a diffuse infiltration of mature plasma cells and lymphocytes within fibrous connective tissue. An immunohistochemical study was performed on formalin-fixed, paraffin-embedded tissues obtained from the skin lesion and from the left cervical node using a peroxidase–antiperoxidase technique. The plasma cells produced various types of heavy chains (γ, μ and α) and both κ and λ light chains. These findings indicated that

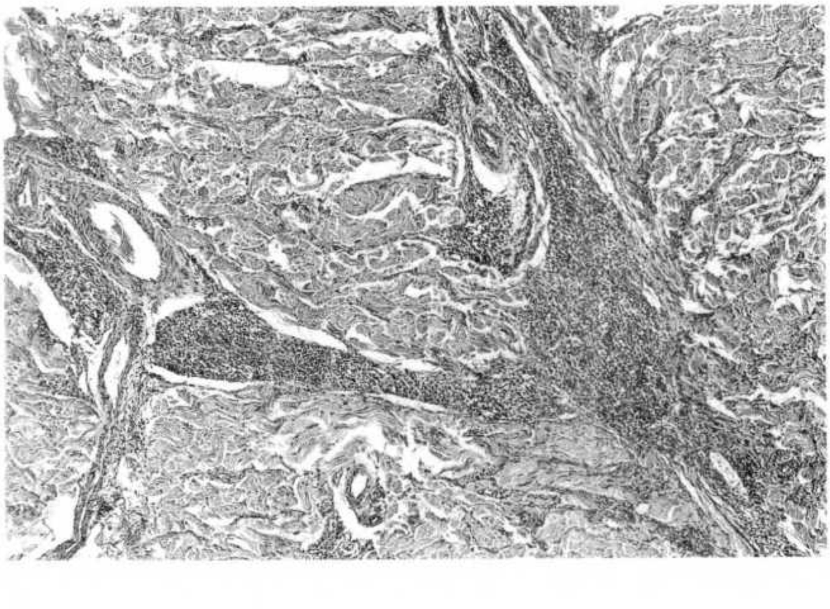

Figure 2. Case 1. Histology of a plaque lesion shows a dense perivascular and periadnexal infiltrate in the dermis (haematoxylin and eosin, × 60).

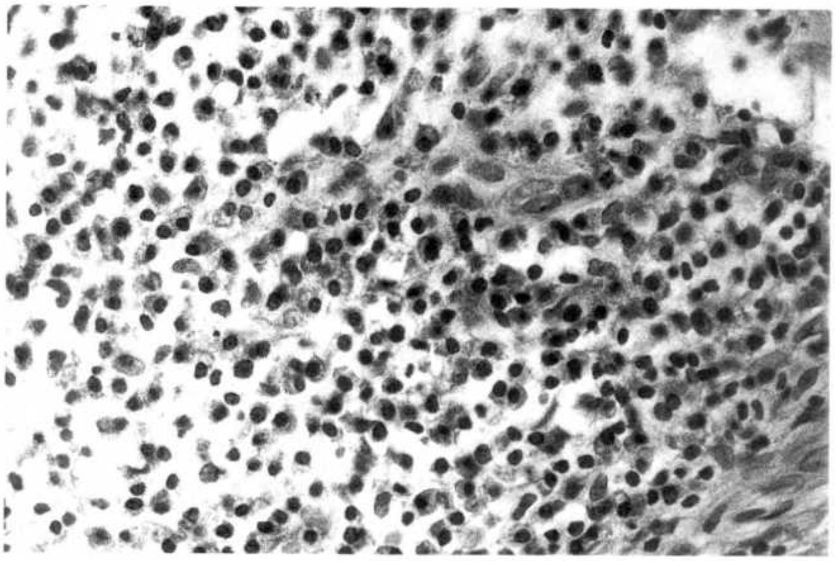

Figure 3. High power view of Figure 2. The infiltrating cells are composed largely of mature plasma cells. These plasma cells display no atypia (haematoxylin and eosin, × 600).

plasma cells showing immunoglobulin production were polycolonal.

The patient was treated with a combination of oral prednisolone (30 mg/day) and cyclophosphamide (100 mg/day) without significant improvement.

Case 2

A 56-year-old man was seen in July 1989 with multiple asymptomatic nodules and plaques of 5 years' duration on the trunk, nape of the neck and limbs. He had been treated intermittently with topical and systemic corticosteroids for years without improvement. The lesions gradually spread and increased in size and number.

Physical examination showed multiple, scattered, well-demarcated, reddish-brown, rubbery hard nodules and plaques, approximately 0·5–3·0 cm in diameter, on the patient's trunk, nape of the neck and limbs (Fig. 4). Superficial lymph nodes, liver and spleen were not palpable. Laboratory values were as follows: ESR, 132 mm/h; white cell count 10,300/mm^3 with 70% segmented neutrophils. C-reactive protein and latex agglutination test were positive. Antistreptolysin O antibody and serological tests for syphilis were negative. Protein electrophoresis showed elevated γ-globulin levels (4·15 g/dl). Serum immunoelectrophoresis showed polyclonal hypergammaglobulinaemia with an IgA of 637 mg/dl; IgG 4,199 mg/dl; and IgM 465 mg/dl. Antinuclear factor and anti-native DNA antibodies were positive at titres of 1:80 and 1:320, respectively. Urinalysis showed no abnormalities and repeated examinations failed to detect Bence Jones protein in the urine. Radiographs of the chest and bones were normal. Computed tomograms of the whole body, ultrasonograms of the abdomen and gallium scinti-photograms were normal. Examination of bone marrow aspirates showed a slightly hypercellular marrow with 78% granulocytes and 2·8% plasma cells. The plasma cells displayed no atypia.

Histology of biopsy specimens taken from plaques on his right thigh and shoulder revealed the same feature as in Case 1. Some of the plasma cells were surrounded by lymphocytes and histiocytes resulting in structures which resembled lymphoid follicles with germinal centres. The plasma cells displayed no atypia. Immunohistochemistry demonstrated that the plasma cells showing immunoglobulin production were polyclonal.

No specific treatment has been given, and there have been no marked changes in the clinical and laboratory findings at the time of writing.

There was no deposition of amyloid in the plasma cell infiltrates in either patient and no underlying disease which might have been responsible for the hypergammaglobulinaemia.

IL-6 studies

Enzyme-linked immunosorbent assay (ELISA) was performed for the quantitation of IL-6. The method has been described elsewhere.[6] The results were expressed as means ± SD. Significant serum levels of IL-6 were detected in both patients (65 ± 2 pg/ml in Case 1 and 23 ± 5 pg/ml in Case 2), whereas IL-6 levels in sera from eight healthy subjects were less than 10 pg/ml or not detectable.

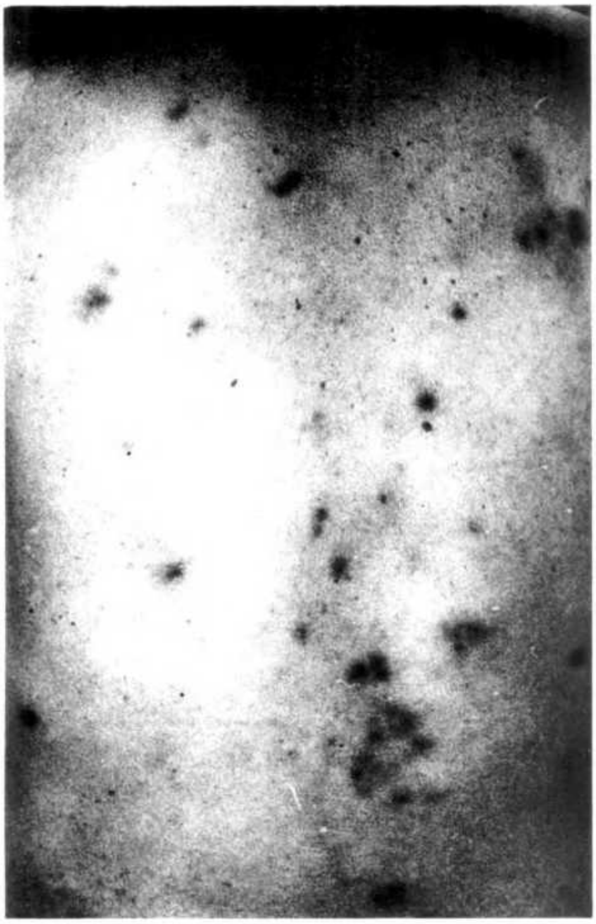

Figure 4. Case 2. Multiple reddish-brown nodules and plaques on the back.

Discussion

In 1956, Castleman *et al.*[7] reported a group of patients with localized benign hyperplastic mediastinal lymph nodes [Castleman's disease or giant lymph node hyperplasia (GLNH)], which Keller *et al.*[8] subsequently subdivided into a hyaline-vascular type and a plasma-cell type, on the basis of histological findings. The plasma-cell type was frequently associated with fever, anaemia, polyclonal hypergammaglobulinaemia, and bone marrow plasmacytosis. The plasma cell type of GLNH may occur in other locations, and in a multicentric form.[9,10] In addition, extranodal lesions in the spleen[11] and lungs[12] have been reported, and were characterized histologically by hyperplasia or infiltration of mature plasma cells. In 1986, Watanabe *et al.*[1] described the association of multiple skin lesions, characterized histologically by prominent hyperplasia of mature plasma cells, with generalized lymphadenopathy and polyclonal hypergammaglobulinaemia, calling this disease complex 'systemic plasmacytosis'. They defined systemic plasmacytosis as a generalized plasmacytosis accompanied by plasma cell proliferation in more than two organs, such as skin, lymph nodes, and lungs, and classified it as a variant of the benign plasmacytoses such as the plasma cell type of GLNH, the multicentric plasma cell type of GLNH, lymphoid interstitial pneumonia with dysproteinaemia (LIP),[12] and idiopathic plasmacytic lymphadenopathy with polyclonal hyperimmunoglobulinaemia (IPL).[13] They realized that proliferation of mature plasma cells localized in some lymph nodes of the body produces the (multicentric) plasma cell type of GLNH; that proliferation in the pulmonary interstitial tissue produces LIP; that extensive proliferation in lymph nodes produces IPL; and that proliferation in more than two organs such as skin, lymph nodes and lungs produces systemic plasmacytosis. It is characteristic of these variants that plasma cells show no atypia, are mature, and polyclonal in nature. The nature of systemic plasmacytosis is thought to be benign plasma cell proliferation, although there may be the possibility of malignant transformation. In addition, cases where mature plasma cells proliferate only in the skin, producing multiple cutaneous nodules or plaques, in association with polyclonal hypergammaglobulinaemia have also been reported, and the term 'cutaneous plasmacytosis' was coined for these by Kimura *et al.*[4]

Our cases had multiple skin lesions composed of mature plasma cells, in association with polyclonal hypergammaglobulinaemia and other abnormal laboratory findings such as increased ESR, and low titres of antinuclear antibodies. In Case 2, there was no clinical or laboratory evidence suggesting involvement of other organs. However, Case 1 had not only multiple skin lesions, but also systemic lymph node enlargement, and multiple peri-ureteric retroperitoneal masses showing diffuse plasma cell infiltration. Extensive investigation of both patients failed to reveal the presence of any underlying disease. Because our patients had antinuclear antibodies, and polyclonal hypergammaglobulinaemia, and showed positive results in tests for C-reactive protein, our cases had to be differentiated from collagen diseases. It would be unusual, however, for the skin lesions observed in our patients to occur in collagen diseases. Therefore, it is a reasonable conclusion that our cases represent variants of the benign plasmacytoses. Clinically and histologically, the features of Case 1 are compatible with a diagnosis of systemic plasmacytosis, because mature plasma cells were proliferating in more than two organs, i.e. the skin, lymph nodes and soft tissue around the ureters, and Case 2 is compatible with a diagnosis of cutaneous plasmacytosis because the proliferation of mature plasma cells was restricted to the skin.

Although the pathogenesis of benign plasmacytoses still remains unclear, Yoshizaki *et al.*[14] demonstrated IL-6 production by cells in the germinal centres of the affected lymph nodes in two patients, one with the plasma cell type of GLNH, and the other with the multicentric plasma cell type of GLNH. In the patient with the plasma cell type of GLNH, clinical improvement and decrease in serum IL-6 were observed following surgical removal of a solitary hyperplastic lymph node. There was a close correlation between the serum levels of IL-6 and the clinical and laboratory abnormalities. Because IL-6 induces B-cell maturation to immunoglobulin-producing cells, it was suggested that production of IL-6 from the affected lymph nodes may be one of the pathogenetic causes of GLNH. This prompted us to determine the serum IL-6 levels of our two patients using an ELISA method. Significant serum levels of IL-6 in both patients suggest that IL-6 may also be involved in the pathogenesis of systemic and cutaneous plasmacytosis. However, IL-6 is produced by various tissues including T cells,[15,16] macrophages,[17] B cells,[18] and keratinocytes.[19,20] IL-6 is present in the serum and urine of renal transplant recipients,[21] in the cerebrospinal fluid of patients with acute infections of the central nervous system,[22] and in the synovial fluid and serum of patients with rheumatoid arthritis.[23,24] Therefore, in order to clarify whether IL-6 production is the primary event in the pathogenesis of benign plasmacytoses, further investigation on additional cases will be needed.

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Mycosis fungoides with a widespread follicular eruption, comedones and cysts

S.OLIWIECKI AND J.ASHWORTH
The Skin Hospital, Chapel Street, Salford M60 9EP, U.K.

Accepted for publication 30 December 1991

Summary We describe a patient with a rare variant of mycosis fungoides (MF) presenting as a widespread follicular eruption with cysts and comedones.

The association of epidermal cysts and MF has been reported previously.[1–5] We present a case in which MF was associated with comedones, cysts and a widespread follicular eruption. A similar clinical presentation has been described in a patient with follicular mucinosis,[6] but there was no evidence of follicular mucinosis in our patient.

Case report

A 61-year-old man presented with an 18-year history of dry scaly skin, initially over the buttocks, but gradually spreading to involve the trunk and limbs. Twelve years ago increasing numbers of cysts and comedones appeared on his face and scalp. Over the past 3 years he has lost most of his scalp hair. The widespread skin dryness gradually developed into a symmetrical follicular hyperkeratotic eruption affecting the trunk and limbs. In areas the follicular lesions coalesced into a more plaque-like appearance. At the same time ulcers on the trunk and limbs have developed periodically which heal with scarring over a period of several months. The clinical features are shown in Figures 1–3.

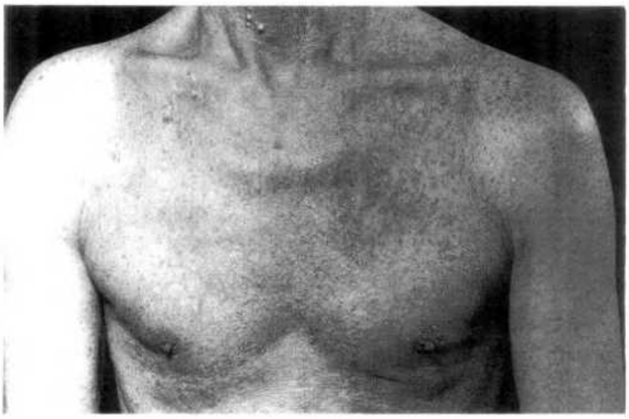

Figure 1. Follicular eruption on chest and arms.

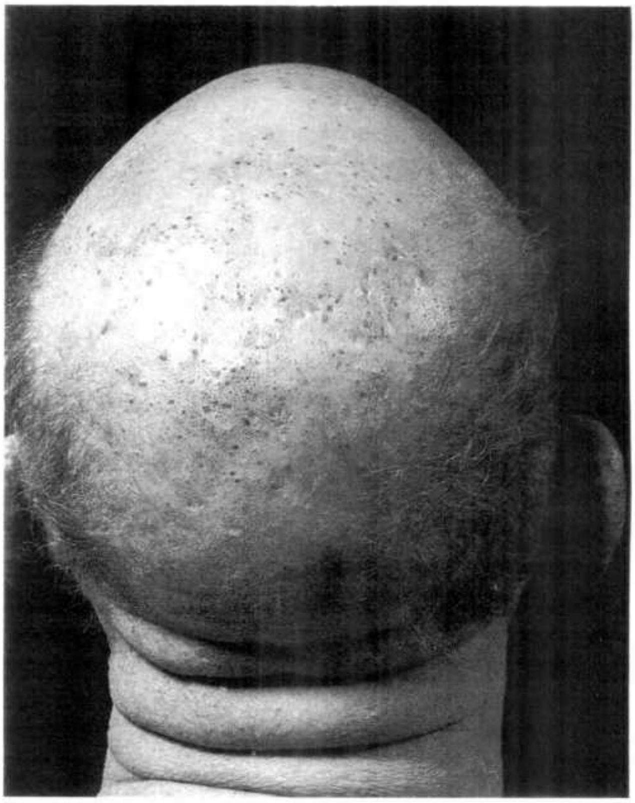

Figure 2. Scalp showing comedones and hair loss.

Skin biopsies were taken in 1989 and 1990. These showed an upper dermal inflammatory infiltrate but there were no diagnostic features. Repeat biopsies taken in 1991 from the forearm, front of chest and back showed the features of mycosis fungoides (Figs 4 and 5). There is an upper dermal infiltrate showing marked epidermotropism, and destruction of the hair follicles, some of which show cystic dilatation. There are no changes of follicular mucinosis and stains for mucin

Correspondence: Dr S.Oliwiecki.

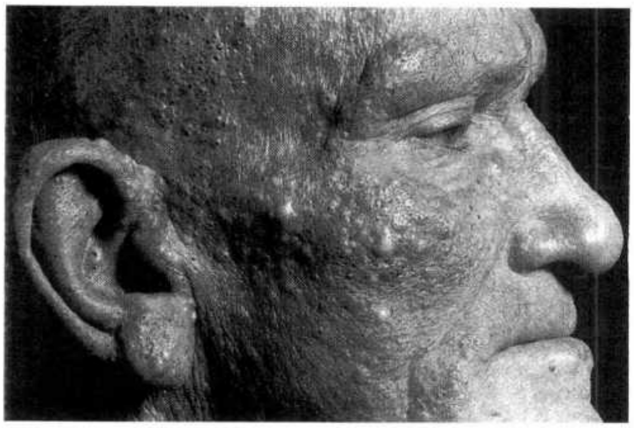

Figure 3. Numerous cysts and comedones on the face.

were negative. Marker studies showed that the cells stained strongly positive with T-cell markers (UCHL 1, Dako). All the sections showed similar histological features although in the specimen taken from the back the changes were less marked and more poikilodermatous.

The disease has shown rapid progression since diagnosis with painful ulceration of the skin in several areas on the trunk and limbs. The patient's general condition has deteriorated and there has been no response to treatment with radiotherapy or etretinate.

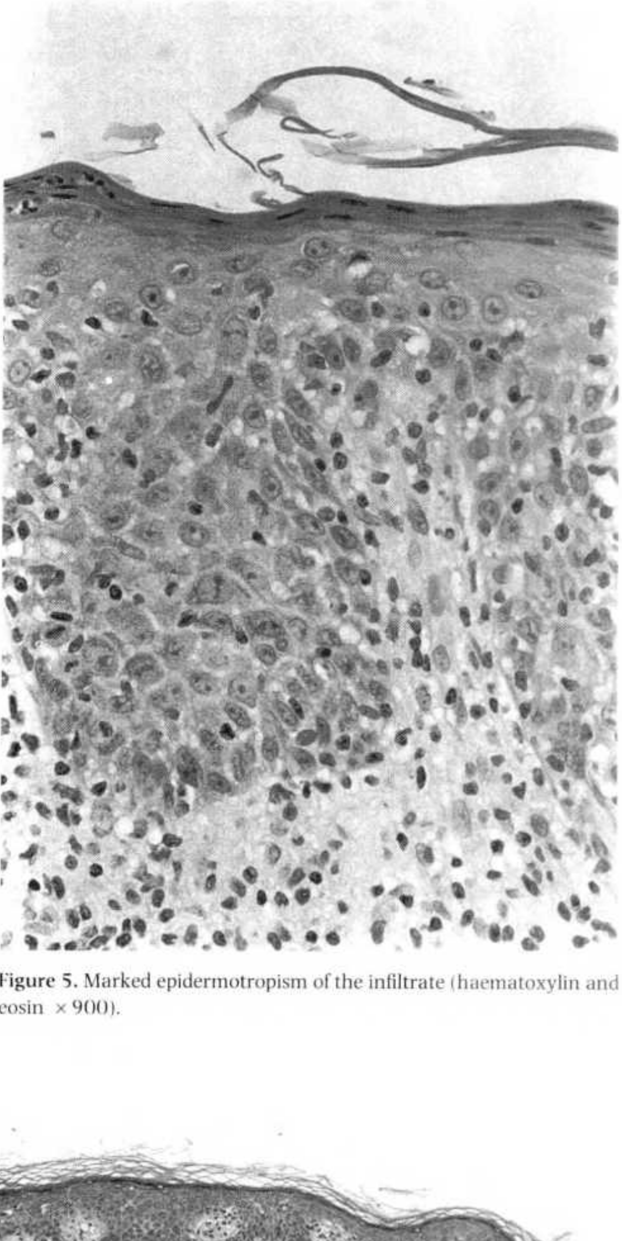

Figure 5. Marked epidermotropism of the infiltrate (haematoxylin and eosin × 900).

Discussion

This patient illustrates a very unusual presentation of MF with a widespread follicular eruption and multiple comedones and cysts on the head and neck. The

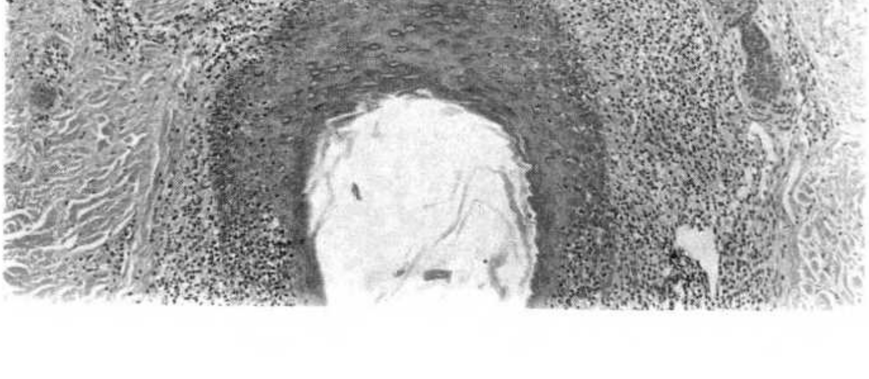

Figure 4. Upper dermal infiltrate with destruction and cystic dilatation of a hair follicle (haematoxylin and eosin × 400).

association of MF and acquired epidermal cysts has been reported previously.[1–5] It has been suggested that MF leads to the development of epidermal cysts by involving and mechanically obstructing the follicular orifices.[2,3] The histology in our case would tend to support this view as it shows destruction of the hair follicle.

Grice *et al.*[6] reported a case of follicular mucinosis and myxoedema with clinical features similar to our case, including facial cysts. The association between follicular mucinosis and MF is well recognized,[7,8] but our patient showed none of the histological features of follicular mucinosis and stains for mucin were negative.

It is unclear why cutaneous T-cell lymphoma should seem to preferentially involve the hair follicles as in our patient. However, it is known that the cell molecules encoded by the major histocompatibility complex (MHC) play an important role in the regulation of T-cell activation.[9] Expression of class II MHC molecules (HLA-DR) in normal skin is limited to the acrosyringial epithelium.[10] However, in alopecia areata HLA-DR expression by follicular epithelium has been described.[11] The peribulbar infiltrates in alopecia areata consist predominantly of T lymphocytes.[12] It has been suggested[11] that aberrant DR expression in alopecia areata may be induced by a variety of non-specific stimuli, for example viral infections, in genetically predisposed individuals. Under certain circumstances this could allow the presentation of autoantigens to helper T cells and give rise to an immune response. It is possible that a similar process may occur in the early stages of T-cell lymphoma and this may in part explain the localization of T-helper cells to the perifollicular area. Further work is, however, necessary to confirm this theory.

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Familial occurrence of crusted (Norwegian) scabies with adult T-cell leukaemia

K.EGAWA,*† M.JOHNO,* T.HAYASHIBARA† AND T.ONO*

**Department of Dermatology, Kumamoto University Medical School, 1-1-1 Honjo, 860 Kumamoto, Japan*

†Department of Dermatology, Yatsushiro General Hospital, 2-26 Matsuejo-machi, 866 Yatsushiro, Japan

Accepted for publication 24 February 1992

Summary We report the occurrence of crusted (Norwegian) scabies in two members of a family, both of whom were suffering from adult T-cell leukaemia (ATL).

Adult T-cell leukaemia, a disease entity first described by Takatsuki *et al.*,[1] is endemic in south-western Japan and the Caribbean islands. The human T-lymphotropic virus type I (HTLV-1) is involved in the pathogenesis of ATL.[2,3]

Clinical manifestions which are not specific for, but are related to, ATL are frequently seen in affected patients, including infectious diseases with unusual clinical features. The non-specific cutaneous disorders in ATL are often extensive, and may be the presenting feature suggesting ATL in endemic areas.[4]

Crusted (Norwegian) scabies is an infestation with *Sarcoptes scabiei* var. *hominis* which is rare, and occurs predominantly in patients with mental deficiency, malnutrition, and immunosuppression.[5] We describe the familial occurrence of crusted scabies in a mother and son who both suffered from ATL.

Case reports

Case 1

A 66-year-old Japanese woman was admitted to the Yatsushiro General Hospital in July 1983, suffering from extensive skin lesions and generalized lymphadenopathy. Her skin was erythrodermic, and there were scattered papules and nodules. Crusted areas were present on her scalp, verrucous hyperkeratotic lesions on the soles of the feet, and the nail plates on both feet were thickened (Fig. 1). Investigations revealed a leucocyte count of $22 \cdot 8 \times 10^9$/l, with 17% abnormal lymphocytes. Anti-HTLV-1 antibodies were positive. The serum lactate dehydrogenase (LDH) was 697 IU/l (normal range 100–300 IU/l); serum calcium 12·4 mg/dl (normal range 8·4–11·4 mg/dl) and serum IgA 272 mg/dl (normal range 140–350 mg/dl). There was diminished response to 'recall' antigens on skin testing. Microscopy of scraping from the scalp, soles of the feet and toenails revealed large numbers of scabies mites. A biopsy specimen taken from a skin nodule revealed small or medium-sized non-Hodgkin's lymphoma of diffuse type. A diagnosis of acute ATL with concomitant crusted scabies was made. There was no history of contact with any other individual suffering from scabies.

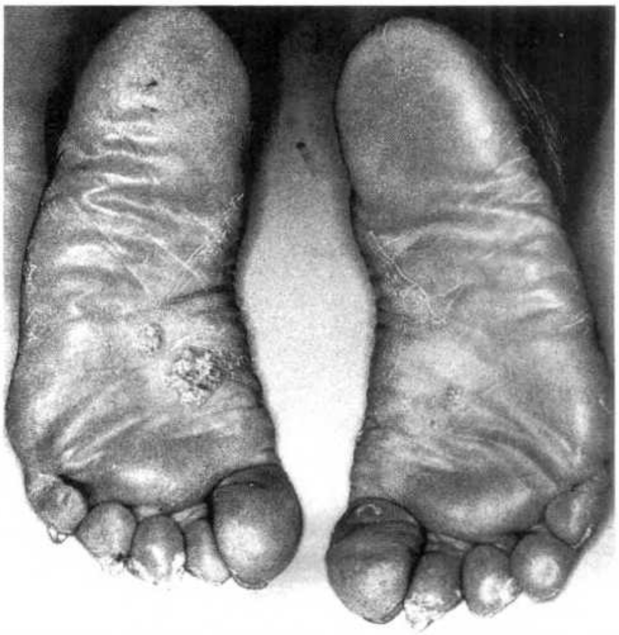

Figure 1. Case 1. Verrucous hyperkeratotic lesions on the soles, and thickened nail plates on both feet.

She was treated with a course of combination chemotherapy (vincristine, adriamicin, betamethasone), with some improvement. The scabies was treated with topical

Correspondence: Dr K. Egawa, Department of Dermatology, Kumamoto University Medical School, 1-1-1 Honjo, 860 Kumamoto, Japan.

10% benzyl benzoate in 70% ethanol with apparent success, but recurrent lesions were occasionally observed during her clinical course. However, in April 1984 she died from rapidly progressive ATL and terminal liver failure.

Case 2

A 40-year-old Japanese man presented to Yatsushiro General Hospital in November 1989 with a 10-month history of crusted areas on the back (Fig. 2), abdomen and penis, and a 4-week history of erythroderma. He was the son of Case 1. However, he had lived apart from his mother and had had no contact with her for 10 years. His general physical condition was good. There was no lymphadenopathy, and no specific cutaneous manifestations of ATL were present. His white cell count was $11 \cdot 3 \times 10^9$/l, with 1–2% abnormal lymphocytes. Anti-HTLV-1 antibodies were positive. Microscopic examination of the extremely thick crust on his back revealed large numbers of scabies mites. A diagnosis of ATL of smouldering type, with concomitant crusted scabies, was made. He gave no history of contact with anyone suffering from scabies. He has not been treated for ATL, but his scabies was effectively treated with topical 10% benzyl benzoate in 70% ethanol. He is presently well.

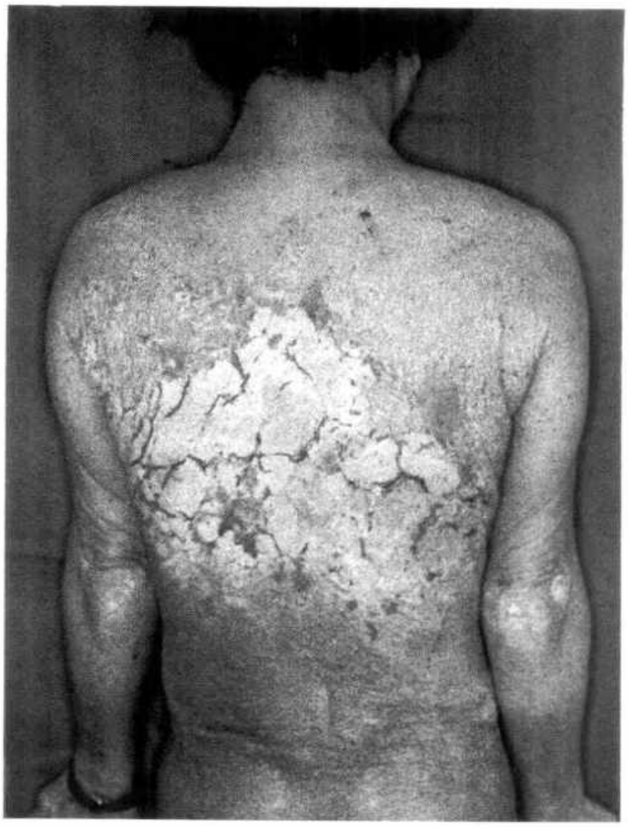

Figure 2. Case 2. Extensive, heavily crusted area on the trunk.

Discussion

Patients with ATL frequently have secondary infections that reflect their diminished cellular immunity,[6] and these may have unusual clinical features.[4] Crusted scabies occurs sporadically in immunocompromised hosts, and it has recently been reported in patients suffering from AIDS,[7] and in association with the acute type of ATL.[8]

Our patients had concomitant crusted scabies and ATL. As they had lived apart, and had had no contact with each other for 10 years prior to the onset of their skin problems, it would appear that they had probably acquired scabies independently. We do not know when or how they were exposed to scabies.

Although some authors,[8,9] have reported low serum IgA levels in patients with crusted scabies, the serum IgA level was within the normal range in Case 1. Our patients had not been treated for ATL before the occurrence of crusted scabies, and the severity of the scabies was assumed to be related to immunosuppression associated with ATL.

The familial occurrence of ATL has been reported. The main route of HTLV-1 infection from mother to child is thought to be via breast milk, and there may be a long time interval before infected individuals develop ATL.[10,11]

Although there has recently been a noticeable increase in the incidence of scabies in areas of Japan where ATL is endemic, crusted scabies remains uncommon. There were no cases of crusted scabies among 500 patients with scabies, and 278 patients with ATL, who have been treated in our hospital over the past 10 years.

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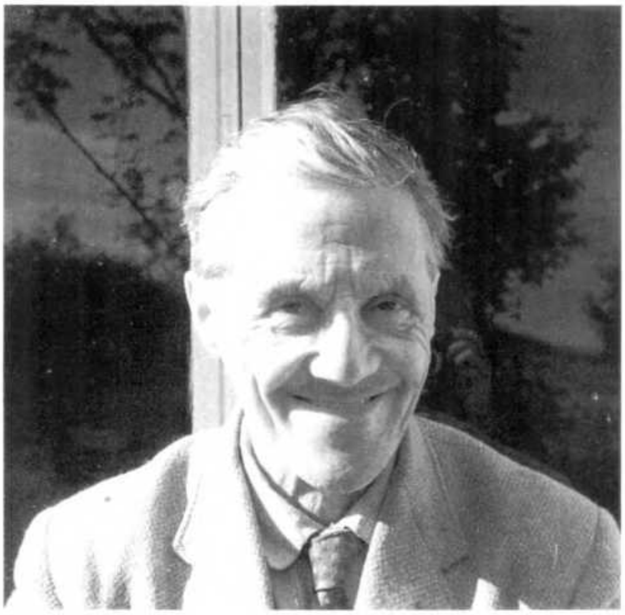
Obituary



Dr Arthur Porter

Dr Arthur Digby Porter, father of British photodermatology, was born on 27 June 1897 at Vevay House, Bray, County Wicklow, Ireland. He died, aged 94 years, on 29 August 1991 at Lamyatt, Somerset. His father had also died at the age of 94 years. Their family home had been in Clogher Park, County Tyrone, Ireland, for well over a century.

In a family of standing such as theirs, preliminary education was by governesses, but the young Arthur would leave lessons to his four sisters, and go fishing with his brother on the River Blackwater, or shooting rats and rabbits. However, Edwardian small boys were required to attend boarding school, and at the age of 8 years he was sent to a prep school in Ireland. He did not like it. After that it was Rugby, Warwickshire, England. He did not like it there either, and entering Pembroke College, Cambridge, in 1915 came as a relief. An honours degree in the Natural Science Tripos (class 2), led to a scholarship at King's College Hospital, London, in 1918. He qualified with the Conjoint Diplomas in 1922 and the Cambridge MA, MB and BChir in 1924. The London MRCP was achieved in 1926, and the Cambridge MD in 1929.

After junior hospital appointments, followed by a few trips as a ship's doctor, he returned to a career in general medicine and held posts in London hospitals and in Essex. He also acquired rooms in Harley Street. An interest in paediatrics was shown by his publications, one in association with Arthur Whitfield, the great teacher and investigator, at King's College Hospital. However, during the next 10–12 years Arthur Porter published nothing and by the end of the 1930s had abandoned Harley Street, general medicine and paediatrics.

In the 1950s and 1960s, it became known that he had suffered from severe duodenal ulcers and haematemeses for many years. Certainly, he observed a strict dietary regime for most of his life. The large thermos flask of gruel which he carried around made him a familiar figure. At the outbreak of the Second World War, his ill-health precluded military service. During the war years, he quietly continued as a consultant at St John's Hospital for Diseases of the Skin, London, where he was appointed in 1939, and the Essex County Hospital, Colchester.

In the early 1950s he developed an important new interest, in sunlight sensitivity, which arose from his work at the Essex County Hospital. This resulted in publications describing the use of a monochromator which he borrowed from a photographic film manufacturer in Ilford, not too far from the County Hospital. It was clear that he was on to something. Looking at his work now, it is evident that he grasped the value of action spectra and that these required not only data from narrow waveband irradiation but also dose–response and time-course studies. He reached these conclusions by himself, for he was, I believe, relatively unversed in scientific method and its literature, for example, he was not aware of H.F.Blum's seminal writings until later. Perhaps his meticulous attitude showed the influence of Arthur Whitfield.

He realized that work on action spectra should help to clarify the many unexplained factors underlying the pathogenesis of solar dermatoses, and he was determined to have a monochromator at St John's in London. The instrument should emit short-wave ultraviolet rays, unlike that in Ilford, and be of much higher power. After many trials, a workable instrument with quartz–water prism and xenon arc was built by Professor W.D.Wright of the Imperial College of Science and Technology, London. The first studies were on patients who had the more common types of solar dermatoses, but work was also performed in collaboration with Professor C.Rimington, on patients with the quite rare metabolic disorder, porphyria. The outcome was the realization that porphyria was more common than previously considered. He also made occasional forays into non-dermatological problems.

The photobiology of human skin had previously been a minority interest and it was entirely owing to the infectious enthusiasm and dogged persistence of Arthur Porter that interest in the subject was awakened in the U.K. He was not the world's first in the field, as others such as H.F.Blum, A.Wiskemann and P.B.Rottier had been involved in the study of photobiology for varying periods of time. It is also to be noted that, coincident with Arthur Porter's pioneering efforts in photodermatology, there had been a growth of interest in photobiology in general in the U.K., culminating in the founding of the British Photobiology Group in 1955.

Arthur had certain traits that favoured success in clinical research, including a ready acknowledgment of his (and everybody else's) ignorance of the underlying processes in dermatological disorders, and a facility for quiet, independent reasoning.

Probably dietary restrictions prevented his attending the junketing of medico-scientific meetings, but there was also, I think, Arthur's shyness. In a large group he would take a back seat and his manner became rather stiff and conventional. However, in a small group he was easygoing, far from conventional and could sparkle with a flow of anecdotes.

On retirement in 1962, the family left their Essex home in Great Easton, because of the building of the third London International Airport at Stansted. By moving to the secluded hamlet of Lamyatt, near Shepton Mallet, Somerset, Arthur was returning to idyllic conditions similar to those of his Edwardian boyhood in Clogher. However, as there was no fishing nearby, he had to travel to Mull, Scotland, where he owned a cottage, to fish, although for salmon fishing he preferred the Spey on mainland Scotland. He was happy in his retirement in remote Lamyatt because he had a special interest in rural life. He got on very well with the neighbours, and in spite of debilitating arthritis, insisted on walking with his two sticks around the village. According to a Sunday newspaper noting his decease, he was a 'shrewd and humorous observer of every detail of village life'.

He is survived by his son, who is a solicitor, a daughter and two grandchildren. His wife, Nina, died in 1984. That they had met was remarkable enough in itself, for she was a Byelorussian, who, caught up in the 1917 revolution, had fled east from near Minsk to Japan, where she remained for 2 years. Yet it was in Surrey that they first met. They married in 1931 in Paris in the old Russian Orthodox Cathedral.

Dr Arthur Porter was by training a clinician whose chosen career was wrecked by ill-health, a situation which led him to take up dermatology. He had little schooling in rigorous science; instead he had an individual way of thinking out things for himself. This led to important contributions which have had wide influence, extending beyond the U.K. Even so, he consistently avoided taking any personal credit for success. This, and his generosity to others, resulted, in part, in his being overlooked, even undervalued, in some quarters during his later professional life. His part in the development of an important line of clinical and investigative dermatology, in the pursuit of which he showed a strong natural talent, should be recognized and remembered.

I.A.Magnus

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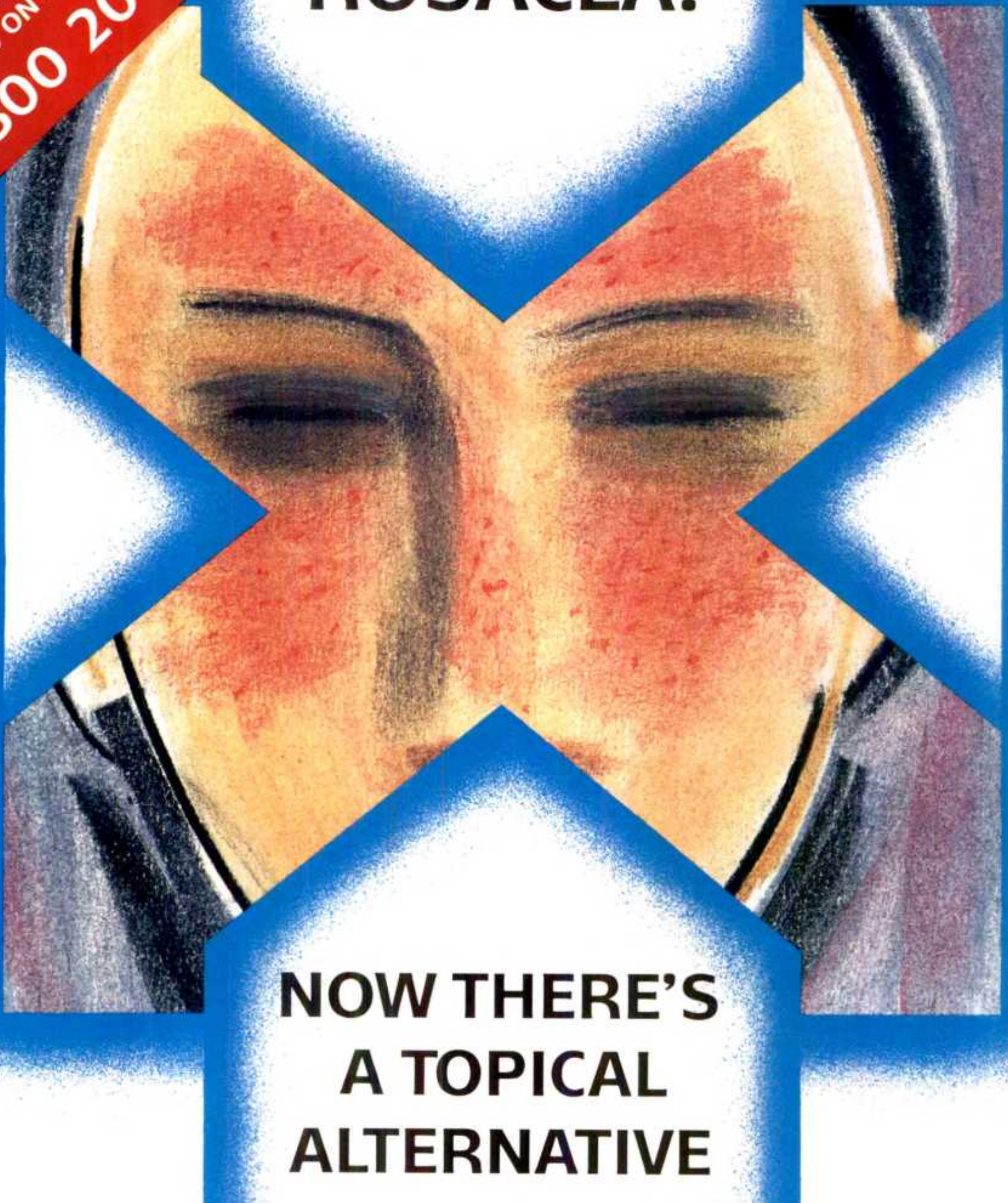

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SANDOZ

British Journal of Dermatology (1992) **127**, 62–67.

Correspondence

Paraneoplastic follicular hyperkeratosis responsive to etretinate

SIR, We have recently seen a patient with striking follicular hyperkeratosis which developed 3 months after diagnosis of adenocarcinoma of the oesophagus, a previously undescribed association. The follicular hyperkeratosis improved rapidly with etretinate and recurred after treatment with etretinate was stopped.

A 72-year-old man was referred with a 19-week history of an hyperkeratotic rash affecting his face and neck. Three months previously the patient had been admitted as an emergency to a surgical ward with a 1-week history of dysphagia. Examination at that time revealed an abdominal mass. The appearances on a barium swallow and on endoscopy suggested carcinoma of the oesophagus. Endoscopic biopsies from the oesophago-gastric junction showed adenocarcinoma-*in-situ*. Abdominal ultrasound revealed diffuse metastatic deposits. A diagnosis of adenocarcinoma of the oesophagus was made. The patient was treated palliatively with laser therapy and was subsequently able to return home. On presentation to the dermatology department he gave a history of having developed abnormal areas of skin affecting his face, neck, scalp, upper chest and back. The eruption was not pruritic. There was no relevant history of skin disease. On examination all the affected areas showed striking follicular hyperkeratosis, with spicules of ketatin protruding from each follicular orifice (Fig. 1). The axillae, groins and palms were not involved. Light microscopy of a skin biopsy taken from the involved skin of the neck showed dilated hair follicles containing discrete protruding columns of hyperkeratosis (Fig. 2). Electron microscopy revealed prominent keratohyalin granules in the keratinocytes of the upper epidermis underlying the hyperkeratotic column. A full blood count showed a haemoglobin of 11·7 g/dl, but other indices were normal. The alkaline phosphatase was raised at 143 IU/l but all other biochemical indices of renal and hepatic functions were normal. Immunoglobulin levels were normal. The patient was commenced on etretinate 25 mg daily. Three weeks after starting this treatment there was a striking improvement in the follicular hyperkeratosis. Two months later due to a deterioration in the patient's general condition, etretinate was stopped, with subsequent relapse of the hyperkeratosis.

A great variety of paraneoplastic cutaneous conditions have been described. These have been divided into obligate, in which a tumour can be expected to be found, and facultative, which are not consistently associated with underlying malignancy.[1] It seems probable that this case of unusual follicular hyperkeratosis was associated with the underlying adenocarcinoma of the oesophagus. Follicular hyperkeratosis has been reported in association with multiple myeloma by a number of authors,[2,3] and in association with carcinoma of the larynx on one occasion.[4] The patient with multiple myeloma reported by Lukitsch *et al.*[2] showed clinical features similar to our patient. However our patient's serum immunoglobulins and electrophoresis were normal, making the possibility of occult mye-

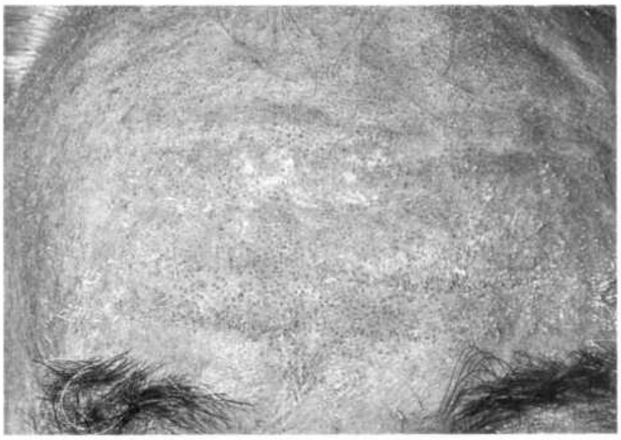

Figure 1. Distinctive follicular hyperkeratosis of the forehead at presentation.

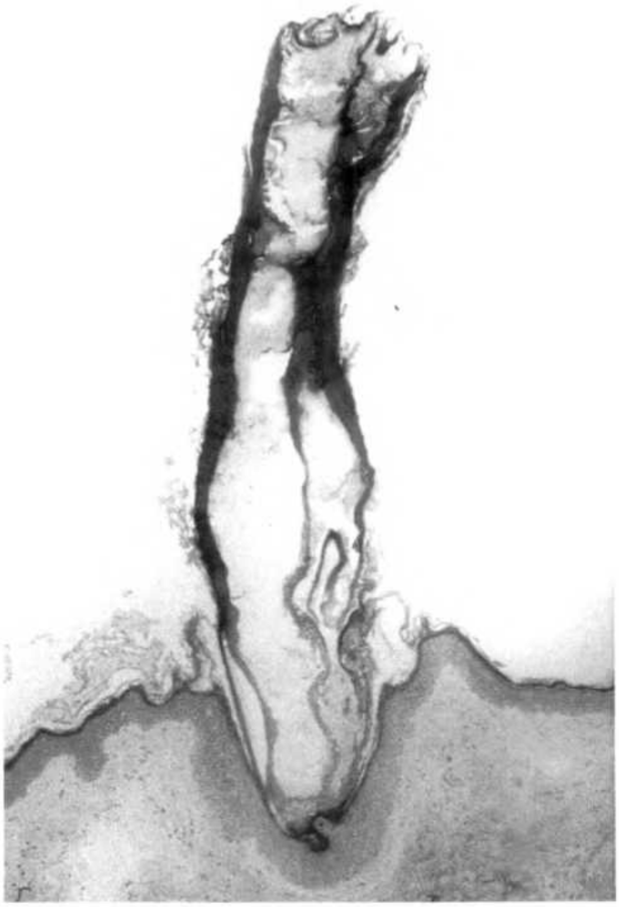

Figure 2. Semithin section of a keratin spicule stained with toluidine blue (Original magnification × 280).

loma very unlikely. A similar acquired follicular hyperkeratosis has been described in the absence of malignancy in malnourished patients,[5] and in hypovitaminosis A.[6] Our patient was reportedly eating and drinking well but was slowly losing weight. It was not possible to determine his exact nutritional status. However, the previous reports of the clinical features of follicular hyperkeratosis in association with non-gastrointestinal malignancy would suggest that these changes may be associated with the underlying condition and are not necessarily directly related to the nutritional status alone. The underlying mechanism that might be responsible for this association is therefore obscure. Specific paraneoplastic syndromes tend to be particularly associated with certain malignancies. Acanthosis nigricans and erythema gyratum repens, for example, are usually associated with adenocarcinoma. The mechanism by which only a minority of patients with a certain neoplasm develop the associated paraneoplastic condition is unknown. We can only speculate that in predisposed individuals who develop certain forms of malignancy, some loss of the normal control mechanisms occur and, in this case, resulted in abnormal follicular keratinization.

University Department of Dermatology, FRANCES HUMPHREYS
The Royal Infirmary, JANE SPENCER
Edinburgh EH3 9YW, U.K. CLAIRE BENTON

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Successful interleukin-2 therapy of advanced cutaneous T-cell lymphoma

SIR, Recombinant interleukin 2 (rIL-2) has been reported to induce tumour regression in some patients with advanced cancer,[1] and there is a report of intralesional treatment of cutaneous T-cell lymphoma (CTCL) with this lymphokine.[2] We have successfully treated a patient with aggressive CD3$^+$, CD8$^+$ CTCL[3] with systemic rIL-2.

CTCL with multiple cutaneous tumours was diagnosed in a 52-year-old man in September 1989. There were no circulating atypical lymphocytes. Histopathological changes were typical of mycosis fungoides. He was in stage IIB (T3 NO MO) according to the classification of CTCL. Dermal infiltrating lymphocytes expressed the following immunophenotype: CD2$^+$, CD3$^+$, CD5$^+$, CD7$^-$ (pan T); CD4$^-$ (T helper); CD8$^+$ (T cytotoxic); HLA-DR$^+$. Less than 1% of lymphocytes expressed the IL-2 receptor Tac (CD25). There was no expression of Ki1Ag (CD30) or Ki67Ag. B cells and natural killer cells (NK) were absent. Successive therapeutic failures included intensive systemic combination chemotherapy and 30 Gy electron-beam irradiation. Autologous marrow transplantation in June 1990 resulted in a complete remission but diffuse cutaneous lesions and systemic symptoms with fever recurred 2 months later.

Interleukin 2 (provided by Roussel UCLAF) was given by continuous intravenous infusion. He underwent three fortnightly courses of 5, 4 and 3 days with 10 MU/m^2/day This induction therapy resulted in rapid improvement, with clearance of over 90% of the skin surface. Complete clinical remission was obtained after five courses of monthly maintenance therapy (10 MU/m^2/day-2 days). No histological sign of mycosis fungoides could be detected in two cutaneous biopsies performed in January 1992. The patient is still in complete remission without any further therapy, 10 months after the last course and 16 months after commencing rIL-2 therapy.

Interleukin 2 is a new immunotherapeutic approach which can result in tumour regression in some patients for whom no other effective therapy is available at present.[1] The treatment of advanced CTCL is unsatisfactory, and no regimen has been shown to be curative. Partial response was obtained in a case of CTCL with intralesional injections of rIL-2.[2] Partial remission was also reported of a large-cell lymphoma of the skin treated with systemic rIL-2.[4] This is the first reported case of complete clinical remission of therapy resistant CTCL with rIL-2 treatment.

Anti-tumour therapy with rIL-2 is thought to act by enhancement of both CD8$^+$ and NK cytotoxic activities and putatively by induction of lymphokine-activated killer cells (LAK). The affected skin was biopsied before each rIL-2 cycle and sequential study of the immunophenotype of the infiltrate was performed. Before the third rIL-2 cycle, the cutaneous infiltrate showed striking changes, with a complete loss of CD2, CD8 and HLA-DR expression and persisting absence of CD4 expression, so the majority of cells appeared negative for both CD4 and CD8. After further courses, a progressive reappearance of the lost markers CD2, CD5, CD7 (unexpressed before treatment), CD4, CD8 and HLA-DR was noted. Before the sixth cycle, the infiltrating cells exhibited a quite 'normal' immunophenotype and were CD2$^+$, CD3$^+$, CD5$^+$, CD7$^+$, CD4$^+$ or CD8$^+$ (with a CD4$^+$/CD8$^+$ ratio$=$1 5), HLA-DR$^+$.

Clearly, rIL-2 warrants studies to determine its place in the therapeutic arsenal against CTCL.

Département de Dermatologie M.RYBOJAD
**Département d'Hématologie,* J.P.MAROLLEAU*
Hopital Saint-Louis B.FLAGEUL
F75475 Paris Cedex 10 M.BACCARD
France M.BRANDELY†
†Roussel UCLAF P.MOREL
102-111 route de Noisy, C.GISSELBRECHT*
93230 Romainville, France

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Capillary leak syndrome in generalized pustular psoriasis

SIR, We were interested to read Dr McGregor's report of two patients with capillary leak syndrome complicating pustular psoriasis.[1] We would like to report a further case with a more favourable outcome.

A 33-year-old obese lady was admitted urgently suffering from widespread erythrodermic psoriasis. Within 12 h of her admission she had developed pustulation of such severity that her skin peeled off in sheets leaving glazed, red, moist areas covering 90% of her body. She had first developed psoriasis with arthropathy in 1981. The psoriasis rapidly became widespread and difficult to control. She had received treatment with PUVA, methotrexate, azathioprine and etretinate with either lack of response or unacceptable side-effects. In 1989 following an acute flare of her psoriasis she was started on cyclosporin A which had been continued at a dose of 2–4 mg/kg/day. Blood pressure and renal function had shown no deterioration and her skin had responded well. Six weeks prior to her admission she had felt vaguely unwell and in the week before admission had felt nauseated, and omitted her cyclosporin for 1–2 days.

On admission her investigations showed normal renal and liver function; her neutrophil count was raised at $22{\cdot}3 \times 10^9/l$ and her chest X-ray was normal. Over the next 3 days her skin improved with continued cyclosporin A and emollients. However, her renal function deteriorated (creatinine 414 μmol/l), her chest X-ray showed fluffy shadowing and her blood pressure fell. She was therefore transferred to the intensive-care unit where she received positive inotropic support and mannitol, and her renal function improved. Her principal problem then became her gas exchange, with progressive hypoxia which was not controlled with continuous positive airways pressure. A decision was made to provide ventilatory support. The fluffy shadowing on X-ray, and the clear frothy fluid from her lungs suggested the diagnosis of the capillary leak syndrome.

At the onset of pulmonary problems blood cultures were negative, with no evidence of Gram-negative sepsis. Later in the course of her illness multiple organisms were isolated from her chest (*Streptococcus pneumoniae*, coliforms) and skin (*Pseudomonas, Staphylococcus aureus*). Antibiotic therapy with vancomycin, gentamicin and erythromycin, was given intravenously in accordance with the sensitivities of the isolated organisms and in addition the skin was treated with topical silver sulphadiazine (Flamazine®). Although her urine output was satisfactory there was a progressive rise in her serum creatinine and urate; she was therefore treated with haemofiltration. Her neutrophil count had also risen progressively reaching $66 \times 10^9/l$. The initiation of haemofiltration coincided with a marked improvement in her skin which had remained erythrodermic despite intravenous cyclosporin A at doses dictated by her blood levels. Her neutrophil count also fell and it was thought that the haemofiltration had probably removed inflammatory mediators such as IL-1 and TNF.

Over the next 3 weeks the patient had a stormy course. Problems included unstable blood pressure, episodes of asystole necessitating resuscitation, and sepsis of both lungs and skin, particularly around intravenous lines. However, 21 days after transfer to the intensive-care unit the patient was extubated, and 3 days later was transferred to the ward; 10 days later she was well enough to be discharged.

Prior to discharge her renal function, full blood count and pulmonary function tests showed minor abnormalities only, and her chest X-ray was clear. She was discharged on cyclosporin A 4 mg/kg/day which was increased to 5 mg/kg/day when her skin flared a week following discharge. Six months after this dramatic episode she remains well.

St John's Dermatology Centre,
St Thomas' Hospital,
London SE1 7EH, U.K.

S.E.HANDFIELD-JONES
M.GARVEY
D.H.MCGIBBON
M.M.BLACK

Reference

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Successful treatment of ulcerating livedo reticularis with infusions of prostacyclin

SIR, Livedo reticularis associated with atrophie blanche (livedo reticularis with ulceration; livedoid vasculitis) is an uncommon condition characterized by purplish discoloration of the skin in a reticular pattern on the legs, leading to painful ulceration, and leaving ivory-white scars after healing.[1] Although this condition has been reported in association with several systemic diseases[2] most cases seem to be idiopathic. The pathogenesis is thought to be related to thrombotic occlusion of small and medium-sized vessels of the dermis. Microscopically deposition of immunoglobulins, complement and fibrin has been demonstrated[3] but inflammatory changes are usually absent.[1–3] Beneficial therapeutic effects have been reported for fibrinolytic agents, heparin, pentoxifylline and antiplatelet drugs[4–8] and are possibly related to the proposed imbalance between coagulation and fibrinolysis in this disorder. However, the response to these treatment modalities is often slow and incomplete, and exacerbations often occur.

Prostacyclin, produced by endothelial cells, has powerful vasodilator and platelet aggregation inhibiting properties, and is in clinical use in conditions such as critical limb ischaemia and Raynaud's phenomenon.[9-11] To our knowledge the effect of prostacyclin infusions in the treatment of ulcerating livedo reticularis has not been reported previously. We report a case of ulcerating livedo reticularis in whom there was a rapid response to treatment with prostacyclin.

A 41-year-old man was admitted to our hospital with painful areas of ulceration on the legs. One year previously he had developed right hemiplegia. Arteriography disclosed occluded branches of the left median cerebral artery and complete occlusion of the left carotid and subclavian arteries close to their origin from the aorta. These radiological abnormalities, the laboratory findings, including a raised erythrocyte sedimentation rate and increased levels of C-reactive protein, and the absence of other signs or risk factors of atherosclerosis, suggested vasculitis of major arteries. He was treated with high-dose corticosteroids at that time.

The following year he gradually developed persistent painful areas of ulceration on the lower legs and feet, surrounded by purplish reticulate discoloration (Fig. 1). There were no signs of chronic venous insufficiency in the legs. Histological examination of the involved skin showed occluded vessels without any signs of vasculitis. Immunofluorescence studies demonstrated deposition of complement C3, IgM and fibrin in the occluded vessel walls as well as intraluminally. Immunohistological examination of a biopsy from normal skin showed no abnormalities. Laboratory tests for cryoglobulin, cryofibrinogen, lupus anticoagulant and anticardiolipin, antinuclear and antineutrophil cytoplasmic antibodies were negative. The clinical picture and the histological findings were compatible with the diagnosis of livedo reticularis associated with atrophie blanche, possibly related to the previous history of vasculitis of major arteries. Because all the lesions were resistant to long-term local therapy, prostacyclin infusions (epoprostenol, Wellcome) were started in a dose of 2 ng/kg/min intravenously 6 h daily. Almost immediately after commencing the infusions the pain disappeared. After 10 days the ulcers showed signs of healing, with granulation tissue formation. Thereafter prostacyclin infusions were given intermittently at weekly intervals for 3 weeks. After 30 days the ulcers were almost healed leaving the typical ivory-white scars of atrophie blanche (Fig. 2).

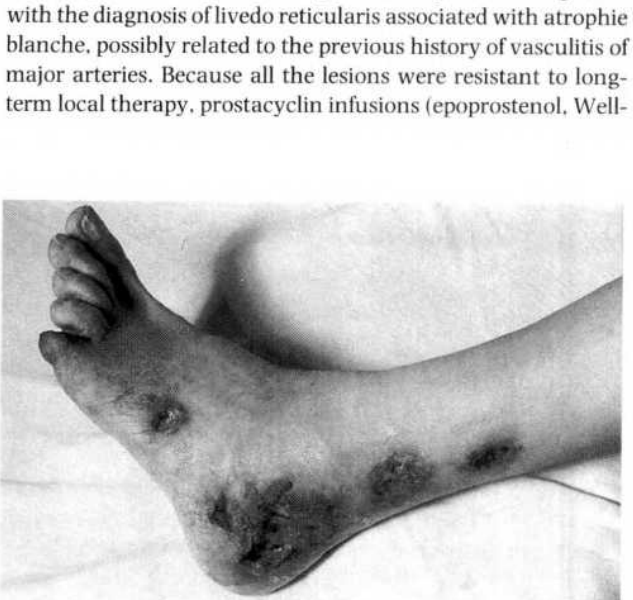

Figure 1. Ulcerating livedo reticularis on the lateral side of the left foot before treatment.

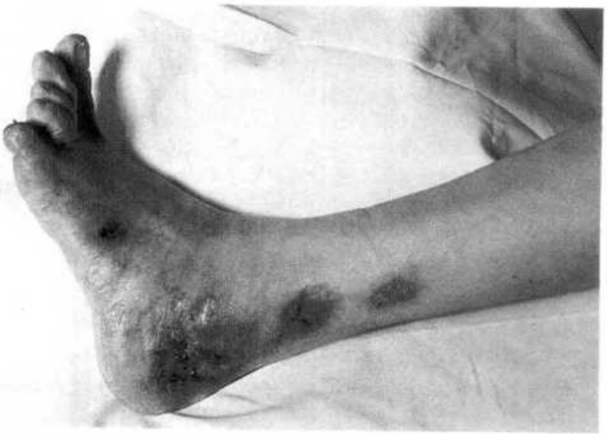

Figure 2. After 30 days of treatment the ulcers were practically healed, leaving the typical scars of atrophie blanche.

In this patient prostacyclin infusions were an effective method of treatment for ulcerating livedo reticularis, and healing was achieved far more rapidly than has been reported with previous therapies. Prostacyclin probably modulates endothelial activation which may be present in this condition. In our opinion prostacyclin should be added to the so far rather limited armamentarium for the treatment of ulcerating livedo reticularis.

Department of Internal Medicine, K.HOOGENBERG
**Department of Dermatology,* R.A.TUPKER*
†Department of Internal Medicine, L.H.VAN ESSEN†
‡Department of Angiology, A.J.SMIT‡
§Department of Clinical Immunology, C.G.M.KALLENBERG§
University Hospital, P.O. Box 30.001,
9700 RB Groningen, The Netherlands

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Allergic contact dermatitis to calcipotriol

Sɪʀ, Calcipotriol ointment provides an effective treatment for psoriasis[1,2] although in one study a facial dermatitis developed in five out of 50 patients.[1] This side-effect was considered to be an irritant reaction to calcipotriol. We wish to report a patient with an allergic contact dermatitis to calcipotriol.

In October 1988, a 50-year-old Caucasian woman with psoriasis was treated with calcipotriol ointment. The treatment was effective, but was stopped after 6 weeks. She then re-started treatment, but this was discontinued after 2 months because of a dermatitis that developed in the areas where the calcipotriol had been applied. The dermatitis cleared after the therapy was discontinued but recurred when the ointment was reapplied.

The patient was patch tested with the European Standard Series, to lanolin, calcipotriol ointment and a placebo ointment, as well as to calcipotriol 50 μg/ml and 10 μg/ml in ethanol (Leo Pharmaceutical Products, Copenhagen, Denmark) and ethanol 67%. After 72 h there was a positive reaction to the calcipotriol, but the placebo ointment and the ethanol vehicle were negative. Calcipotriol in ethanol in concentrations of 50 μg/ml and 10 μg/ml were also positive. The patient was again patch tested several months later with calcipotriol solutions and with 50 μg/ml and 10 μg/ml of vitamin D_3 (calcitriol) in ethanol, vitamin D_2 (ergocalciferol, 25-hydroxy vitamin D_3 and 1-α-hydroxy vitamin D_3 in ethanol. In addition to positive reactions to calcipotriol, there were also weak positive reactions to 25-hydroxy vitamin D_3 and 1-α-hydroxy vitamin D_3 (Table 1).

Table 1. Results of different patch-test reactions with calcipotriol ointment, placebo ointment, and vitamin D analogues in 67% ethanol.

	Patch-test reactions		
Patch-test materials	I	II	III
Calcipotriol ointment	+		
Placebo ointment	–		
Calcipotriol 50 μg/ml	+	++	
Calcipotriol 10 μg/ml	+	++	
25-OH.D3 50 μg/ml		–	?+
25.OH.D3 10 μg/ml		?+	+
1-α-OH.D3 10 μg/ml		?+	+
Ethanol 67%	–	–	–

Our patient had an allergic contact dermatitis to calcipotriol and had positive reactions to other vitamin D analogues, 1-α-hydroxy vitamin D_3 and 25-hydroxy vitamin D_3. As with corticosteroids, cross-sensitivity appears to depend upon small molecular differences[3,4] and hydroxylation at different points in the molecule is of importance.[4]

Our findings and those of Yip and Goodfield[5] show that calcipotriol can elicit contact allergy in man, and this should be considered when a patient with psoriasis develops a dermatitis while on treatment with this vitamin D analogue.

Department of Dermatology, D.P.Bʀᴜʏɴᴢᴇᴇʟ
Free University Academic Hospital, C.W.Hᴏʟ*
De Boelelaan 1117, C. Nɪᴇʙᴏᴇʀ
NL-1081 HV Amsterdam,
The Netherlands
**Leo Pharmaceutical Products,*
PO Box 51, NL-1380 AB,
Weesp,
The Netherlands

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Severe lichen planus clears with very low-dose cyclosporin

Sɪʀ, Clearance of lichen planus with 3–5 mg/kg daily of cyclosporin has been described.[1,2] There is evidence that a much smaller systemic dose may be effective in oral lichen planus, as the response described in some studies of high doses of topical cyclosporin given as a mouthwash[3,4] may well be due to systemic absorption of a small amount of the preparation.[5]

We have studied the effect, in an open pilot study, of low-dose oral cyclosporin, initially 1 mg/kg body weight daily, in four patients with severe symptomatic cutaneous lichen planus unresponsive to potent topical corticosteroids (Table 1). Two patients also had erosive oral lesions. Assessments were made every 2 weeks and the dose increased every 4 weeks by 50 mg daily if new lesions or itching were still present. When the oral and skin disease was completely clear the cyclosporin was discontinued.

Table 1. Patient characteristics

Sex/age (years)	Duration of disease	Symptoms	Body sites involved	Dose (mg/kg) to clear	Follow up
F/56	6 months	Itch preventing sleep	Thighs, back, hands	1	Few lesions at 3 months
F/34	9 years	Severe itch	Forearms, flanks, axillae, thighs, feet, mouth	2·5	Few lesions at 1 week
F/54	2 months	Walking painful	Hands, feet, back, mouth	1	Clear at 6 months
F/63	6 months	Walking painful	Hands, feet	2	Clear at 6 months

Two patients cleared after 4 weeks on 1 mg/kg daily, one patient after 6 weeks on doses up to 2 mg/kg daily and one patient after 11 weeks on doses up to 2·5 mg/kg daily. All patients remained normotensive and blood tests showed no change in the full blood count, in renal or hepatic function, or urate levels. All blood trough cyclosporin levels were under 100 ng/ml, and some levels were undetectable (less than 25 ng/ml by radioimmunoassay) on the 1 mg/kg dose, when clinical improvement had occurred. However, all patients had detectable levels (38–95 ng/ml) at doses at which they finally cleared. One week and 3 months, respectively, after stopping cyclosporin two patients developed further skin and oral lesions which were less severe than the original lesions and could be controlled with topical corticosteroids. The other two patients remained clear 6 months after stopping treatment.

The fact that in these patients severe lichen planus unresponsive to topical corticosteroids cleared with 1–2·5 mg/kg of oral cyclosporin daily means that further controlled studies of very low doses are indicated.

University Department of Dermatology,
Royal Victoria Infirmary,
Newcastle upon Tyne, U.K.

N.J.LEVELL
C.S.MUNRO
JANET M.MARKS

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British Journal of Dermatology (1992) 127, 68–69.

Book Reviews

Rook/Wilkinson/Ebling Textbook of Dermatology. 5th edn. EDITED BY R.H.CHAMPION, J.L.BURTON AND F.J.G.EBLING (1991). Oxford: Blackwell Scientific Publications. Pp. 3570. Illustrations 1767. ISBN 0-632-02396-1. Price £275.00.

It is a measure of the importance and prestige of this book that anyone asked to write a review for a leading journal would consider it a very considerable compliment. This book was first published in 1968 and with each new edition there have been substantial improvements and changes to the book. However, these have been so subtle that, not unlike a Mercedes, while considerable changes and improvements occur with a new model, it maintains a recognizable and enduring quality, so that those who have grown used to it and love it will not feel uncomfortable with the changes, and many who have not used it before will find changes which will attract them. It was therefore with great interest that one waited to see what changes had occurred in the new 1991 edition. There are many that will be instantly recognizable: an increase in the number of pages from 2167 to 3570, an increase in the size of the pages, four volumes instead of three, the retirement of two of the original editors, Dr Rook and Dr Wilkinson, and extensive use of colour illustrations.

Dr Rook, unfortunately, died before the public launch of the book but he must have been, as I am sure Dr Wilkinson will be, pleased with what he saw. Dr Champion and Dr Burton have taken over the major clinical editorial responsibility. Four authors have dropped out but 21 new authors have been added so that there are now 53 contributors. However, the editors have clearly kept a very tight discipline over their contributors and have produced a uniform format for each chapter, consisting of sections on aetiology, pathology, clinical features and treatment. With commoner diseases there are additional sections, including definition, incidence, prevalence, course and prognosis, differential diagnosis and associations. Anyone who has edited a book, no matter how small, will realize the problems in producing a uniform text in a multiauthor book. The editors have clearly succeeded in what must have been a very difficult task. This greatly adds to the value of the book and the ease with which it can be used as a reference text, which is its main function. I cannot imagine anyone would sit down and read the book through, although there may be some brave souls who will do this. It is not meant to be used in this way. Nevertheless, it is so readable that I am sure many will read complete chapters. There are other subtle changes in the book which those who have used the previous editions will find helpful. These include display of the page numbers on the spine of each volume, the use of bolder type in the index to highlight the pagination of sections containing the principal description of a disorder, and the larger type face which the page size allows. All of these add to its usefulness as a quick reference book. The change from black and white photographs to colour is a major advance, although as often happens, there is an occasional photograph which may lose something in the change. However, this is very much the exception. The photographs are small, as in previous editions, and none is full page, but this is in keeping with the philosophy that the book is primarily a text and not a colour atlas. Where photographs are included, they are relevant and illustrate the point for which they are included. The provision of full reference details with titles and full pagination for each reference is another useful and helpful change, particularly in a textbook which is rewritten on average every 6 years. The authorship is primarily British but the editors have not hesitated to seek expertise elsewhere if they felt it would add to the book. There is no doubt that the group of authors collected by the editors would be considered by dermatologists in Britain to be the leaders in their fields, and to have the respect of their colleagues as such.

There has been a considerable rearrangement of existing chapters, and new chapters including Comparative Dermatology, Anatomy and Organization of Human Skin, Functions of the Skin, and Histopathology of the Skin—General Principles, have been added. The chapter in previous editions on tumours of the skin has been subdivided and there are now separate chapters on epidermal skin tumours, tumours of the skin appendages, soft tissue tumours, and melanocytic naevi and malignant melanoma. In keeping with the times, there is now a chapter on physical and surgical therapy, and an increase in the section on AIDS from 1 to 18 pages—in addition to coverage in the sections on Kaposi's sarcoma, psoriasis, seborrhoeic dermatitis and T-helper cells. Another good example is the complete reorganization of the section on aplasia cutis congenita, now called 'Congenital absence of skin', and increased from 2 to 6 pages.

Some might consider the price a deterrent, but with very few exceptions, for example those practising in very specialist fields, the shelves of any dermatologist could be completely cleared and their contents replaced by this book. It is complete in depth and width and covers the whole field of dermatology from anthropology to zoonoses. Bearing this in mind, and with the quality of production, the price per page is very sound value.

I have always wondered how anyone could practise dermatology without this book. In any department it should be at every point of duty, be it out-patients, the ward, paediatric clinic or operating room. I have no doubt that this new edition will be consulted more frequently, and will be even more useful, more essential and more successful.

D.BURROWS

Cutaneous Surgical Anatomy of the Head and Neck. E.A.BREISHCH AND H.T.GREENWAY (1992). London: Churchill Livingstone. Pp. 100. Illustrations 60. ISBN 0-443-08744-X. Price £18.50.

This is one of an excellent series of eight manuals covering the discipline of dermatological surgery. The authors have taken a regional approach to the head and neck with 11 chapters covering each region, such as the ear, the nose, the eyelid, etc.

in detail. The format of each chapter is similar with, where relevant, details of surface anatomy, muscles, blood supply and lymphatics, motor nerves, sensory nerves and clinical considerations. This latter section brings to life the otherwise 'sterile' aspect of gross anatomy.

Every page has a large, clear line drawing relevant to the text. When dealing with the anatomy of such a complex area as the head and neck on a regional level, the relationship between each area may be lost, but two colour plates at the beginning of the book overcome this.

The text is clear and concise, details only major structures and does not cover any small variations in anatomy that may and, of course, regularly do occur.

An exception to the regional approach is a whole chapter devoted to the factial nerve. This is a very valuable chapter detailing the course of the facial nerves in the face and the sites at which they are at greatest danger of severance with resultant motor paralysis.

This is a neat and concise book and its contents should be thoroughly understood by any dermatologists who perform cutaneous surgery on the head and neck.

S.I.White

Writing Successfully in Science. Maeve O'Connor (1991). London: Harper Collins Academic. Distributed by Chapman and Hall. Pp. 229. ISBN 0-04-445806-1 PB; 0-04-445805-3 HB. Price: £8.95 PB; £25.00 HB.

The 'publish or perish' ethic has led to an ever increasing volume of scientific literature. Some contributions to journals provide significant additions to knowledge, some are interesting to read, and a few are well-written. A substantial number of manuscripts received by long-suffering editors, however, are carelessly prepared, poorly presented, and written in a manner which suggests only a passing acquaintance with the mother tongue. It can be argued that it is the scientific content, not the literary style, which is the most important aspect of a scientific communication, but there is no doubt that the overall presentation has a profound influence on referees, editors and the eventual readership. It therefore behoves the writers of scientific papers to expend some effort in presenting their material in a manner likely to impress rather than repel. Maeve O'Connor's book provides a comprehensive guide to achieving this aim.

The first 11 chapters are a completely revised version of *Writing Scientific Papers in English* (O'Connor and Woodford, 1975), and cover all the steps involved in preparation of a manuscript for publication. The reader is provided with an expert guide to writing a scientific paper, including advice on layout, referencing, presentation of tables and illustrations, aspects of style and grammar, submission of the manuscript, and proof reading. The remaining chapters cover the preparation of short talks and posters, writing a thesis, writing review articles and book reviews, and preparation of grant proposals and curricula vitae—all packed with sound practical advice. The appendices contain a transcript of the 'Uniform requirements for manuscripts submitted to biomedical journals' (reproduced from the *British Medical Journal*, 1988), and a useful list of suggested terms to replace long-winded, unnecessarily verbose phrases in the text of a manuscript. The presentation is clear and concise, and the style is eminently readable.

This book is a gem which should be read and re-read by anyone contributing to the scientific literature, and by editors of scientific journals. It is a writers' and editors' *vade-mecum*. I would advise purchase of three copies of the book—one to take pride of place with a dictionary and thesaurus next to the word processor in your study, a second for your departmental library, and a third to replace the second when it 'walks' into a passing briefcase on indefinite loan. It would be difficult to imagine a book which would provide better value for money.

D.A.Burns

British Journal of Dermatology (1992) 127, 70.

News and Notices

Dermatopathology at Lake Constance with A.Bernard Ackerman, M.D., 8–10 October 1993

This course consists of three full-day sessions, including self-assessment courses, on Histological Diagnosis of Inflammatory Skin Diseases—A Method by Pattern Analysis. Topics to be discussed will include: New Patterns of Inflammatory Skin Diseases; Superficial Perivascular Dermatitis; Superficial and Deep Perivascular Dermatitis; Vasculitis; Nodular and Diffuse Dermatitis; Intra- and Subepidermal Vesicular Dermatitis; Folliculitis and Perifolliculitis; Panniculitis.

For registration and information contact: Dermatopathology Laboratory, Heino Hügel, M.D., Heinz Kutzner, M.D., Arno Rütten, M.D., Siemensstr. 6/1, W-7990 Friedrichshafen 5, Germany.

The skin disease research fund: strategy for the 1990s

The Skin Disease Research Fund is a nationally based charitable organization, set up in 1973 by the Institute of Dermatology (now St John's Institute of Dermatology), devoted to fund raising, with the object of supporting research on the aetiology, pathophysiology, diagnosis and treatment of skin diseases. In recent years the Fund's strategy has been to act as a source of grant support supplementary to the MRC and the Wellcome Trust, rather than an alternative to these bodies. Furthermore, the Trustees have hitherto been reluctant to undertake even short-term commitments to staff salaries. However, the Trustees have recently re-evaluated the role of the Fund taking into account the present climate for obtaining MRC and Wellcome grant support. It is widely recognized that over the past few years even 'alpha' rated proposals frequently remain unfunded by the MRC and the situation at Wellcome is almost as difficult. Other smaller charities are in similar difficult circumstances. The Trustees have decided, for the time being, to modify the Fund's strategy and encourage proposals for more substantial projects equivalent, for example, to 3-year project grant support. It is hoped that the Fund will be able to support one or two such projects annually. It is further hoped that this will not exclude consideration of smaller pump-priming or supplementary proposals as in former years, although this will depend upon the level of future donations to the Fund.

The recent 1991 round of awards were nine in number, totalling £142,888. These included a grant of £33,947 to Dr M.Rustin of the Royal Free Hospital for research on the pathogenesis of atopic dermatitis, £28,656 to Dr Fenella Wojnarowska of the Slade Hospital, Oxford for work on linear IgA disease and £25,885 to Dr A.Chu of the Hammersmith Hospital for research on Langerhans cell function.

The conduct of the research itself remains the exclusive responsibility of the recipient of the award, but it is a condition of each award that the Trustees be kept in regular touch with the progress of the research. For this reason individual members of the Grants Advisory Committee will be assigned to each project for liaison purposes.

The current members of the Grants Advisory Committee are Professor Malcolm Greaves (Chairman), Sir Colin Dollery, Professor John Turk, Professor Stanley Bleehan, Professor Terrence Ryan and Professor John Hunter.

A call for grant applications is normally advertized in the *British Journal of Dermatology* in the summer each year, proposals being considered in November and December. Applications will only be considered at other times under exceptional circumstances.

Readers of the BJD and especially members of the BAD are asked to do what they can to seek additional donations to the Fund by personal contact and by displaying posters and leaflets in clinic areas and waiting rooms. These and other information can be obtained from: the Secretary, The BAD Office, 3 St Andrew's Place, London NW1 4LB. Telephone 071-935-8576.

The Skin Disease Research Fund

The Fund invites further applications for grants. The deadline for submissions is 4 September 1992 and an indication of the result will be given during November. Only the Fund's own forms will be accepted and these may be obtained from: The BAD Office, 3 St Andrew's Place, London NW1 4LB. Telephone 071-935-8576.

Third International Conference on Melanoma, 31 March–3 April 1993, Venice, Italy

The Third International Conference on Melanoma will take place at the G. Cini Foundation, S. Giorgio Island, Venice on 31 March–3 April 1993. The deadline for the receipt of abstracts is 31 August 1992. For further information please contact: Dr Mario Santinami, Secretary General, c/o Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian, 1-20133, Milan, Italy. Telephone 39/2/2663992 or 26680626. Fax 39/2/26680636.

British Journal of Dermatology (1992) **127**, 71–78.

REVIEW

Vitamin D analogues and psoriasis

J.BERTH-JONES AND P.E.HUTCHINSON
Department of Dermatology, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

Accepted for publication 10 April 1992

Summary Topical vitamin D analogues offer a new, effective, more convenient and generally well-tolerated option for the treatment of psoriasis. Only psoriasis vulgaris has been intensively studied, but other forms of the disease may also respond. Both calcitriol and calcipotriol have been shown to be effective in numerous clinical trials, and the latter has compared well with betamethasone valerate and short-contact dithranol in controlled studies. Their mechanism of action is not yet fully understood and may prove complex. The most important effect may be a direct regulation of keratinocyte proliferation and differentiation. However, these compounds also have potent immunological properties, and may act by inhibition of cytokine production by keratinocytes or lymphocytes. Topical application of vitamin D analogues appears generally to be remarkably safe, but hypercalcaemia and hypercalciuria may develop if large quantities are used.

In 1991 the first topical vitamin D analogue (calcipotriol, calcipotriene, 50 μg/g in an ointment base, Dovonex®, Daivonex®), was licensed in the U.K. for the treatment of psoriasis vulgaris. This review encompasses current evidence regarding the efficacy, mode of action and safety of vitamin D analogues.

Vitamin D and psoriasis

Vitamin D became available in commercial quantities in 1930 when vitamin D_2 (calciferol, ergocalciferol), was manufactured by irradiation of ergosterol derived from yeast. Another compound found in animal fat became known as vitamin D_3, or cholecalciferol. The latter can be produced in the skin by ultraviolet (UVB) irradiation of 7-dehydrocholesterol which is synthesized by the keratinocyte. It has been estimated that an area of skin of just 20 cm^2 exposed to the sun for 3 h daily,[1] or an oral dose of vitamin D_3 of just 10 μg daily, will prevent rickets.

Cholecalciferol requires activation by two hydroxylation reactions catalysed by a hepatic 25-hydroxylase, and a renal 1-hydroxylase to produce the active hormone 1,25 dihydroxycholecalciferol (calcitriol). The production of this hormone in the kidney by the final 1-hydroxylation is regulated in response to the parathormone level, the plasma phosphate level, and by its own level. Excretion of vitamin D and its metabolites is mainly by hepatic secretion into the bile.

During the 1930s and 40s vitamin D was prescribed for a very wide variety of diseases. Responses were claimed in conditions as diverse as pemphigus vulgaris,[2] scleroderma,[3] and tuberculosis.[4] Reports appeared of psoriasis responding to large (and toxic) oral doses.[5,6]

A further link between calcium metabolism and psoriasis came from numerous reports of the disease flaring or becoming pustular during episodes of hypocalcaemia. In some cases this hypocalcaemia may have been secondary to the hypoalbuminaemia caused by the severe psoriasis, but in others it was clearly due to malabsorption, or hypoparathyroidism.[7–10]

These empirical observations were followed by a series of scientific advances. Vitamin D analogues were found to interact with specific high-affinity nuclear receptors.[11] These were identified in many cell types including keratinocytes, dermal fibroblasts, monocytes and activated lymphocytes.[11–14] Investigation of these receptors demonstrated that interaction with their ligands promoted keratinocyte differentiation. and inhibited proliferation.[15,16]

Efficacy of vitamin D analogues in psoriasis

Interest was stimulated in 1985 by a Japanese report of striking improvement in psoriasis in a patient treated for osteoporosis with oral 1α-hydroxyvitamin D (alpha-

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Correspondence: Dr J.Berth-Jones.

calcidol).[17] These investigators later reported a number of open trials on a total of 52 subjects with psoriasis among whom responses were observed to oral alphacalcidol 1 μg daily, oral calcitriol 0·5 μg daily and topical calcitriol 0·1–0·5 μg/g.[18–20]

A series of trials performed in the United States involving a total of 124 subjects again demonstrated response of psoriasis to both oral and topical calcitriol.[13,21] These investigators used the drug systemically at doses starting with 0·25 μg daily and increasing until hypercalciuria appeared. Topical calcitriol proved effective in placebo-controlled, double-blind studies in ointments containing 3 and 15 μg/g.

In the United Kingdom smaller studies have shown responses to both oral alphacalcidol and topical calcitriol.[22,23] Additional placebo-controlled, double-blind studies from Japan[24] and Poland[25] have confirmed the response to topical calcitriol. Okano[24] was able to demonstrate an effect with a concentration as low as 0·1 μg/g.

The degree of clinical improvement in these studies was variable, and two double-blind trials showed no response to topical calcitriol.[26,27] Both of these used a solution of the drug in medium chain triglyceride containing 2 μg/ml. However, taking the results together there can be no doubt that a response occurs, and this is closely related to dose and concentration.

In order to minimize effects on calcium metabolism whilst retaining therapeutic activity in psoriasis other vitamin D analogues have been investigated. Topical 1,24 dihydroxycholecalciferol appeared effective in an open study,[28] but this compound is almost as active as calcitriol in mineral metabolism.

Attention has therefore recently focused on calcipotriol, and this has now become the most intensively investigated vitamin D analogue for the treatment of psoriasis. Calcipotriol has less than 1% of the activity of calcitriol on calcium homeostasis.[29]

The first published study on the use of this compound was a double-blind, placebo-controlled, right/left comparison using a cream formulation.[30] Twenty-seven subjects applied vehicle alone to one side of the body and an active cream containing one of three concentrations of calcipotriol to the other, twice daily for 6 weeks. There was a clear dose-related improvement on active treatment. In a further trial of similar design, three concentrations in an ointment formulation were compared.[31] The response was again significantly better on active treatment than on placebo, and better at 50 than at 25 μg/g, but did not improve further at 100 μg/g.

A third placebo-controlled study demonstrated an unequivocal response to topical calcipotriol at 6 weeks using the higher concentration of 1·2 mg/g.[32]

In a large, European multicentre study of double-blind, right/left design, calcipotriol ointment (50 μg/g) was compared with betamethasone valerate ointment (0·1%) in 345 subjects.[33] Each treatment was applied twice daily for 6 weeks and response was assessed using the psoriasis area and severity index (PASI). There was a significant difference in favour of calcipotriol at 2, 4 and 6 weeks.

A parallel-group, double-blind comparison between calcipotriol and betamethasone valerate in the same formulations has recently been completed.[34] Four-hundred and five patients participated and response was again monitored using the PASI. Both treatments produced a highly significant benefit. There was no significant difference in improvement of PASI between the two treatments, although patients considered calcipotriol more effective.

Calcipotriol has been compared with short-contact dithranol therapy in a multicentre, open, parallel-group study.[35] Two well-matched groups, each of 239 subjects with chronic plaque psoriasis, were treated for 8 weeks. There was a greater reduction in PASI on calcipotriol than on dithranol, and subjects rated calcipotriol significantly more acceptable than dithranol.

A considerable amount of investigation has now been performed relating to long-term use of topical calcipotriol. In the largest long-term study yet published, 167 subjects participated and the duration of treatment was 12 months.[36] The mean PASI fell from 8·1 to 2·7 over the period of the study. Fifteen subjects were withdrawn due to deterioration of the psoriasis. Fifty complained of some irritation or other cutaneous adverse effect over the 12-month period, but only nine withdrew as a result. No subject developed hypercalcaemia.

Research has already begun on combining calcipotriol with other treatments. In a right/left comparison 20 patients used calcipotriol alone on one side of the body and combined with ultraviolet irradiation (UVB) on the other.[37] Only a small benefit was demonstrated from the addition of UVB.

Both calcitriol and calcipotriol have therefore been shown to be effective when used topically in placebo-controlled studies. Calcipotriol is at least as effective as 0·1% betamethasone valerate or short-contact dithranol therapy, whilst being better tolerated than the latter. In the majority of patients, the beneficial response can be maintained by long-term treatment.

Mechanism of action of vitamin D analogues in psoriasis

Vitamin D analogues appear to function in a manner similar to other groups of steroids. They combine with highly specific nuclear receptors which regulate gene transcription by binding, in the presence of a nuclear accessory factor (NAF), to a specific sequence of DNA, known as the vitamin D response element (VDRE).[38] In addition, more immediate 'non-genomic' effects are believed to operate through different mechanisms. These include elevation of intracellular calcium,[39] and cyclic GMP levels.[40]

The presence of the vitamin D receptor in keratinocytes, fibroblasts and immunologically active cells such as macrophages and activated lymphocytes raises a question as to which cell is the principal target through which calcitriol and its analogues act in psoriasis.

In vitro studies have demonstrated a potent ability of calcitriol to inhibit cell proliferation and promote differentiation in both mouse[15] and human[13,16] keratinocyte cultures. These properties have also been demonstrated in numerous other benign and malignant cells lines, including skin fibroblasts,[13,41] breast cancer cells,[42] leukaemia cells[43,44] and histiocytic lymphoma cells.[29] Several other vitamin D analogues,[45–47] including calcipotriol,[29,48] also demonstrate these actions. Calcitriol may also promote both transcription and release of TGF-β by human keratinocytes, and this cytokine suppresses keratinocyte proliferation synergistically with calcitriol.[49] The further observation that expression of the vitamin D receptor is up-regulated in hyperproliferative epidermis,[50] would strongly support the hypothesis that the keratinocyte is the primary target cell for topical calcitriol analogues in psoriasis. Against this, there are reports that both keratinocytes[51] and fibroblasts[52,53] from psoriatic skin are relatively resistant to inhibition of proliferation by calcitriol, although this seems to vary from patient to patient.[13]

Psoriasis is known to respond to cyclosporin, a potent inhibitor of lymphocyte activation. Calcitriol and its analogues share this property, suggesting an immunological mechanism in which the lymphocyte or antigen-presenting cell may be the primary target. The diverse actions of vitamin D analogues include inhibition of lymphocyte activation by interleukin 1,[54,55] inhibition of interleukin 2 production,[55–57] inhibition of immunoglobulin synthesis,[58,59] inhibition of TNF production by monocytes,[60] and inhibition of lymphocyte chemotaxis.[61]

In vivo effects of vitamin D analogues on the skin have been investigated using a variety of methods. In two studies the effect on epidermal proliferation after tape stripping was measured, with conflicting results. In the first no inhibition of proliferation was demonstrated,[62] although the formulation employed was the same as that used unsuccessfully at the same centre to treat psoriasis.[27] In the second, there was marked inhibition of proliferation.[63]

Normalization of the epidermal cytokeratin profile during treatment with vitamin D analogues has been demonstrated by various techniques. Keratin electrophoresis on skin biopsies obtained during treatment with oral alphacalcidol demonstrated that expression of keratins K16 and K18, both markers of hyperproliferation, returned to normal levels whilst K2, a marker of differentiation, increased to a level higher than normal.[22] Similar changes were observed by the same method during treatment with topical calcipotriol,[64] and a reduction in K16 expression and DNA content of epidermal cells has been demonstrated by flow cytometry.[65]

This evidence would indicate than *in vivo*, as *in vitro*, vitamin D analogues inhibit keratinocyte proliferation and promote differentiation, and would therefore suggest that the keratinocyte is a primary target cell. However, histological studies have yielded evidence that immunological changes may also play a role.

De Jong *et al.*[66] found that the earliest change, present at 1 week, was a marked reduction in intensity of polymorph infiltration of the epidermis. This was followed by a reduction in cycling epidermal cells (expressing Ki 67) at 2 weeks, a reduction in epidermal and dermal T lymphocytes by 4 weeks and a relatively small reduction in K16 expression even after 12 weeks. Macrophages fell slightly in both compartments, whilst epidermal Langerhans (T6$^+$) cells increased.

Two studies have compared histological changes during treatment with calcipotriol and betamethasone valerate. Mallet *et al.*[67] found that histological features were normalized to a similar extent on both treatments, but no reduction was seen in expression of K6 or K16. Berth-Jones *et al.*[68] found that K16 was markedly reduced by both treatments, and the effect of betamethasone appeared slightly greater than that of calcipotriol. However, the latter had a more potent effect on K5 and K10, which were used to measure restoration of normal differentiation. There were also marked reductions in T4$^+$ and T8$^+$ lymphocytes on both treatments. T6$^+$ cells fell rapidly on betamethasone valerate but less so on calcipotriol, and, in some subjects, increased on this treatment. Keratins K5 and K10 each responded to calcipotriol to a greater degree than epidermal thickness, whereas each component of the infiltrate appeared to 'lag behind'.

Expression of IL-6 and TNF-α have been examined using polyclonal antibodies. Calcipotriol reduced keratinocyte expression of IL-6 (a possible regulator of epidermal proliferation), but not TNF-α.[69]

These investigations are consistent with a primary effect of vitamin D analogues on keratinocyte proliferation and differentiation. In seems very likely, however, that the immunological properties of these compounds also play a role.

How safe are topical vitamin D analogues?

Changes in calcium homeostasis

Overdose of vitamin D leads to hypercalcaemia as a result of increased calcium absorption from the gut and resorption of bone. Symptoms which may occur include malaise, headaches, drowsiness, constipation, polydipsia, polyuria, muscle weakness, fatigue, irritability, nausea, and vomiting. Chronic hypercalcaemia may result in urinary stones, soft tissue calcification in blood vessels, myocardium, and cornea, nephrocalcinosis and renal failure.

When activated (i.e. 1-hydroxylated) forms of vitamin D such as calcitriol and calcipotriol are used, the physiological control mechanism has been bypassed and there is a risk of hypervitaminosis if use of these compounds is indiscriminate. Conversely, the ability to compensate by reducing endogenous production of calcitriol may provide something of a safety factor.

Evidence from the clinical trials described above certainly confirms that oral vitamin D preparations increase serum calcium levels and urinary calcium excretion. Administration of these compounds late at night (when there is less calcium in the bowel), in conjuction with a low calcium diet, may allow larger doses to be given safely.[70]

The results from topical treatment are more reassuring. In virtually all the clinical trials described above, either serum or urine calcium or both were monitored. With the exception of a case reported below, the only evidence of any disturbance in calcium metabolism during topical treatment with any vitamin D analogue was seen in patients treated with calcitriol by Langner *et al.*[25] These investigators found no overall increase in mean serum or urinary calcium during their study. However, among subjects using the highest concentration of calcitriol yet investigated (15 μg/g applied twice daily) three patients developed transient asymptomatic hypercalcaemia, and in the group with the largest treatment area urine calcium excretion was slightly increased.

Calcipotriol has less than 1% of the activity on calcium metabolism of calcitriol.[29,71] The most likely explanation for this selectivity of action is the rate of elimination. In the rat, the area under the plasma concentration/time curve for calcipotriol is less than 1% of the area for calcitriol.[72] This may be due to a lower affinity for serum vitamin D binding protein.[73] Human hepatocytes metabolize calcipotriol *in vitro* in the same manner as those of the rat, by oxidation to a much less active 24-keto derivative.[74]

The extensive clinical research performed on topical calcipotriol has indicated a high degree of safety. Serum calcium was monitored during all the clinical studies performed on this compound and none has shown a significant rise in the mean level. Only limited data are available regarding urine calcium excretion during calcipotriol treatment. Three studies, on a total of 69 subjects, have failed to demonstrate any increase,[75–77] even in long-term use,[77] in patients using less than 100 g of ointment weekly. No changes have been detected in serum calcitriol, parathormone or 25-hydroxycholecalciferol levels.[64,74,75]

However, intoxication may result if excessive quantities are used. A case of asymptomatic hypercalcaemia developed during a clinical trial using calcipotriol.[34] A subject with widespread psoriasis used, against instructions, 400 g of ointment (50 μg/g) in 10 days. The serum calcium rose from 2·29 mmol/l at baseline, to 2·73 after 4 days and 3·4 mmol/l at 10 days. In a further case, a patient with impaired renal function used 200 g of calcipotriol ointment over 7 days for the treatment of exfoliative psoriasis involving 40% of the body surface.[78] The patient developed nausea, muscle weakness and abdominal pain and the serum calcium rose to 3·51 mmol/l. Both cases recovered within a few days of discontinuing treatment.

Two further cases of hypercalcaemia have been reported among 19 patients with advanced breast cancer treated with topical calcipotriol.[79] This would seem much more likely to have been a complication of advanced malignancy than an adverse effect of the treatment.

On current evidence, monitoring of serum or urine calcium does not appear necessary if less than 100 g of calcipotriol ointment (Dovonex®) is used weekly. However, if quantities above this limit are required it is essential to monitor the serum calcium closely. Twenty-four hour urinary calcium excretion is a more sensitive parameter than the serum level because increased calcium absorption results in increased excretion before the serum calcium rises.[21] The investigation is highly dependent on adequate compliance by patients who must provide complete collections. Urinary calcium/creatinine ratios provide a more convenient, but less precise alternative.

Other adverse effects

Irritant reactions have been reported in up to 19·5% of patients using calcipotriol in short-term studies.[34] These are not usually severe. In a large trial in which 345 subjects used calcipotriol, only two (0·6%) required this treatment to be discontinued due to irritation.[33] The face is a particularly frequent site for these reactions.[31] Sensitization has also been reported but is much more unusual.[80] Neither irritant reactions nor sensitization have so far been reported with topical calcitriol.

The antiproliferative effects of vitamin D analogues, which they share with corticosteroids, may give rise to concern over the possibility of inducing cutaneous atrophy. Although this has not been seen in patients using topical calcitriol for 3 years,[81] there is a need for more assessment of this risk.

Calcitriol and calcipotriol stimulate differentiation of osteoclasts from their precursors.[82] There is therefore a theoretical risk that vitamin D analogues may promote bone resorption. However, the actions of vitamin D on bone are complex, because both compounds also increase osteoblast activity.[83] As discussed above, urine calcium excretion does not usually appear to rise during topical treatment, so that significant demineralization of bone seems unlikely to occur.

Overall, on current evidence, topical calcipotriol would appear to be remarkably safe even in long-term use, provided that the maximum of 100 g/week is not exceeded. Irritant reactions are frequent, but rarely severe.

The future

Evaluation of vitamin D analogues in psoriasis has so far been largely confined to psoriasis vulgaris. However, trials are already underway to investigate the response to calcipotriol in scalp psoriasis and palmoplantar pustulosis. All clinical research has so far been conducted on adults, but two trials on the treatment of children with calcipotriol were commenced in the autumn of 1991. Results of further long-term studies on large numbers of subjects will soon become available.

A scalp application and a cream formulation of calcipotriol are being developed and should be available soon. Trials are underway to assess the benefit and safety of using occlusion with this compound. More research is required on combination therapies. Initial experience in Leicester would indicate that added benefit is obtained when calcipotriol is used with cyclosporin, PUVA, methotrexate and etretinate. It may prove possible to reduce doses of second-line treatments in this way.

Vitamin D analogues are already being investigated in a range of other diseases. No response was found in preliminary studies on ichthyoses,[24] alopecia totalis,[84] or acne vulgaris (W.J.Cunliffe, personal communication).

A large range of further vitamin D analogues are currently under investigation. Some are potent in promoting differentiation and inhibiting cell proliferation.[45,85] Others may prove to be particularly effective immunosuppressants.[55,86] Applications for these may extend well beyond the treatment of psoriasis.

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British Journal of Dermatology (1992) 127, 79–84.

Dermatofibrosarcoma protuberans is a unique fibrohistiocytic tumour expressing CD34

SETSUYA AIBA, NOBUKO TABATA, HIROTADA ISHII,* HARUO OOTANI† AND HACHIRO TAGAMI

*Departments of Dermatology, *Orthopedics and †Pathology, Tohoku University School of Medicine, 1-1 Seiryo-Machi, Sendai, 980, Japan*

Accepted for publication 13 February 1992

Summary

Dermatofibrosarcoma protuberans (DFSP) is a slow growing, locally invasive tumour whose differentiation from other fibrohistiocytic tumours sometimes poses serious diagnostic problems. We investigated CD34 expression immunohistologically in various fibrohistiocytic tumours including dermatofibroma, DFSP, malignant fibrous histiocytoma (MFH), infantile myofibromatosis, fibrosarcoma, hypertrophic scar and keloid. Among these, DFSP was unique in that tumour cells themselves expressed CD34, whereas in other tumours, CD34 expression was observed only on vascular endothelial cells amongst the tumour cells. Until now, there have been no reports of useful immunohistological markers for DFSP. CD34 expression by the tumour cells can be an extremely useful marker in establishing a definitive diagnosis of DFSP.

Dermatofibrosarcoma protuberans is a spindle-cell tumour which may sometimes be difficult to differentiate histologically from other fibrohistiocytic tumours such as dermatofibroma, fibromatosis and malignant fibrous histiocytoma. It is important to distinguish DFSP from dermatofibroma because their biological behaviour differs greatly. In contrast with dermatofibroma, DFSP grows in a more infiltrative fashion, and possesses a greater capacity for local recurrence, although it rarely metastasizes. The differentiation of DFSP from a typical dermatofibroma confined to the dermis does not usually pose any problem. However, when the fibrous variant of dermatofibroma, composed predominantly of densely packed fibroblasts, extends into the subcutaneous tissue, it can be difficult to distinguish from DFSP. There have been several immunohistological studies of DFSP,[1–7] including the report by Cerio *et al.*,[5] suggesting that factor XIIIa staining is helpful in distinguishing DFSP from dermatofibroma. DFSP is factor XIIIa negative, whereas dermatofibroma is factor XIIIa positive. With regard to differentiating DFSP from dermatofibroma extending into the subcutaneous tissue, Kamino and Jacobson[8] recently presented histological criteria to distinguish between these entities.

The histiogenesis of DFSP is still a matter of debate. Almost 25 years ago neuroectodermal origin was a popular hypothesis.[9,10] Several authors later supported this view with electron microscopic evidence that suggested origin from a modified neural supporting cell, such as the perineural cell.[11,12] Today, many authors favour either a histiocytic,[13–15] a fibroblastic,[16–21] or a dual fibroblastic–histiocytic origin.[22,23] Recent immunohistochemical study strongly supports a fibroblastic histogenesis of DFSP.[4,6]

Nickoloff[24] has recently reported the presence of a distinctive population of human progenitor cell antigen (CD34) positive dermal spindle-shaped cells. These CD34-positive cells are present in a perivascular and interstitial distribution, and are more numerous in the reticular than the papillary dermis. They are also present in the basement membrane zone of the eccrine ducts and secretory glands, and in the perifollicular area of the mid-portion of the follicle known as the bulge area. Another population of dermal spindle-shaped cells are CD34 negative and are distributed in the papillary and reticular dermis. The ratio of CD34 positive to negative spindle-shaped interstitial cells in the reticular dermis ranges from 1:9 to 3:7.

In this paper, we examined CD34 expression on several fibrohistiocytic tumours, and found that DFSP was unique among these in its expression of CD34. CD34 expression may present a novel method of differentiating DFSP from other fibrohistiocytic tumours, including

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Correspondence: Dr Setsuya Aiba.

dermatofibroma, and suggests that DFSP originates from CD34-positive dermal spindle-shaped cells.

Methods

Formalin-fixed, paraffin-embedded tissue sections from seven clinically and histologically proven cases of DFSP, 18 cases of dermatofibroma, nine cases of malignant fibrous histiocytoma, two cases of infantile myofibromatosis, two cases of hypertophic scar, two cases of keloid, and one case of fibrosarcoma were stained by an avidin–biotin peroxidase technique, using anti-CD34 antibody (anti-human progenitor cell antigen, HPCA-1, Becton Dickinson Co. Mountain View, CA, U.S.A.; 2 μg/ml in final concentration), anti-macrophage antibody (anti-CD68, KP-1, Dakopatts A/S, Denmark), isotype control (mouse IgG_1 control, Becton Dickinson Co.: 2 μg/ml), anti-human von Willebrand factor antibody (Dakopatts) (1:400), anti-factor XIIIa antibody (Dakopatts) (1:400), and non-immune rabbit serum (1:400). The 18 cases of dermatofibroma comprised 10 cellular and eight fibrous dermatofibromas. In eight cases the dermatofibroma extended into the subcutaneous tissue. The distinction between dermatofibroma and DFSP was based on the criteria of Kamino and Jacobson.[8] Among their criteria, we principally utilized the following: dermatofibroma—the presence of a perivascular infiltrate with occasional multinucleated histiocytes,

Table 1. Immunohistochemical staining of fibrohistiocytic tumours

Diagnosis	Number of cases	CD34	Factor VIII	Factor XIIIa	CD68
Dermatofibrosarcoma protuberans	7	+	–	–	–
Dermatofibroma	18	–	–	+	–
Malignant fibrous histiocytoma	8	– (+ in one case)	–	– (+ in one case)	–
Infantile myofibromatosis	2	–	–	–	–
Hypertrophic scar	2	–	–	–	–
Keloid	2	–	–	–	–
Fibrosarcoma	1	–	–	–	–

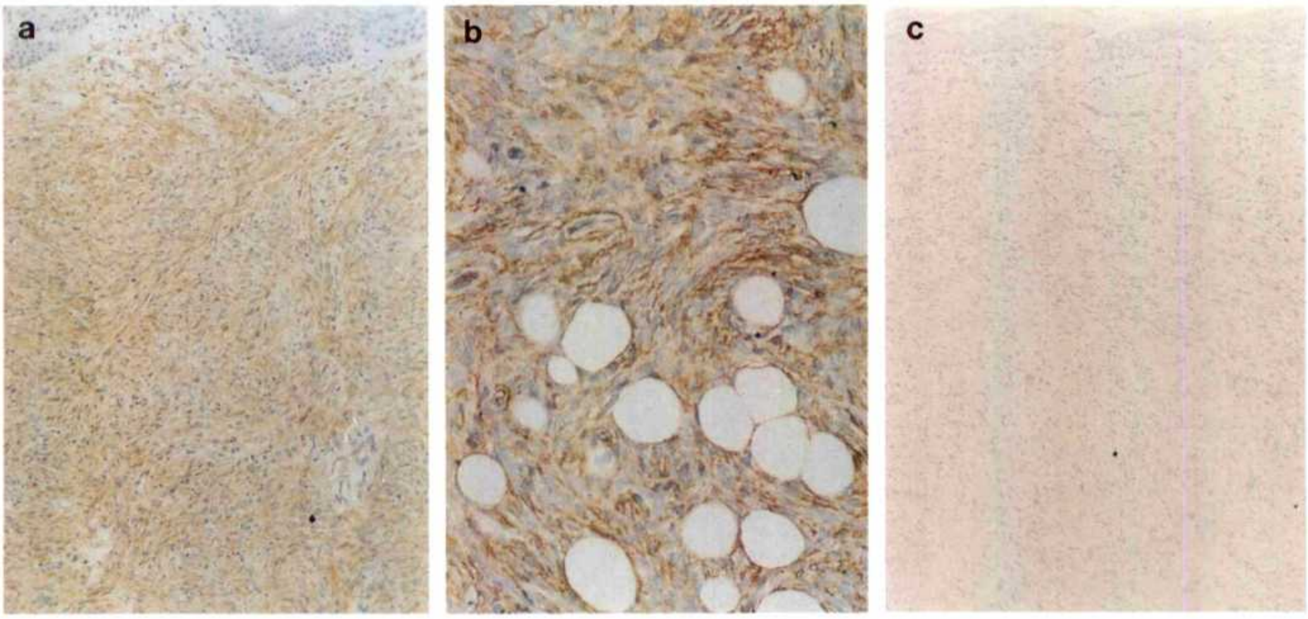

Figure 1. Immunoperoxidase staining of dermatofibrosarcoma protuberans for CD34. (a) Low-power view: the tumour cells extending from the upper dermis to deep dermis reveal CD34 staining (× 100). (b) High-power view: the tumour cells infiltrating into subcutaneous tissue in a lace-like pattern reveal membranous CD34 staining (× 300). (c) Low-power view: isotype-matched control antibody does not stain tumour cells (× 100).

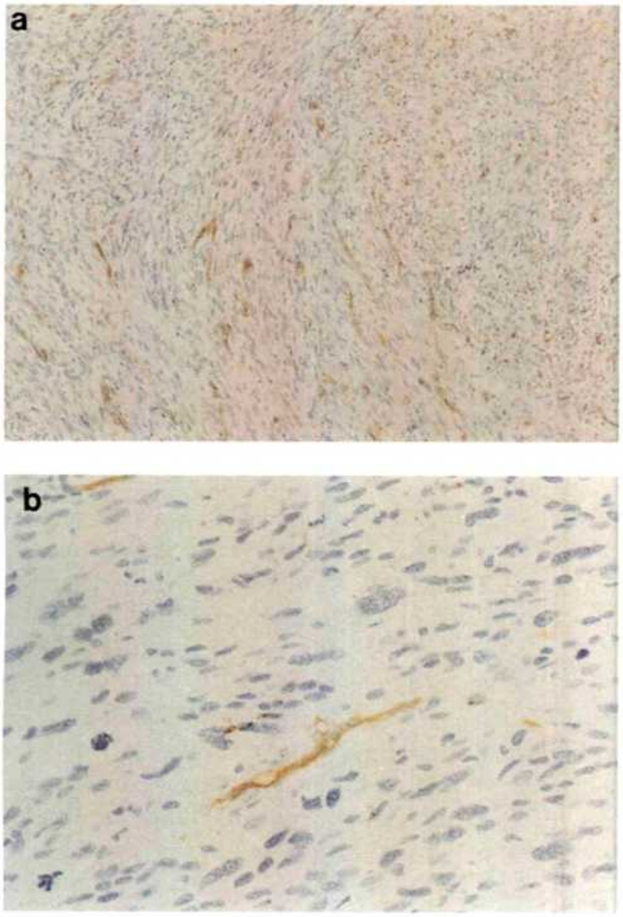

Figure 2. Immunoperoxidase staining of dermatofibroma and malignant fibrous histiocytoma. (a) In dermatofibroma only endothelial cells amongst the tumour cells reveal CD34 staining (× 100). (b) In malignant fibrous histiocytoma, only endothelial cells amongst atypical tumour cells reveal CD34 staining (× 400).

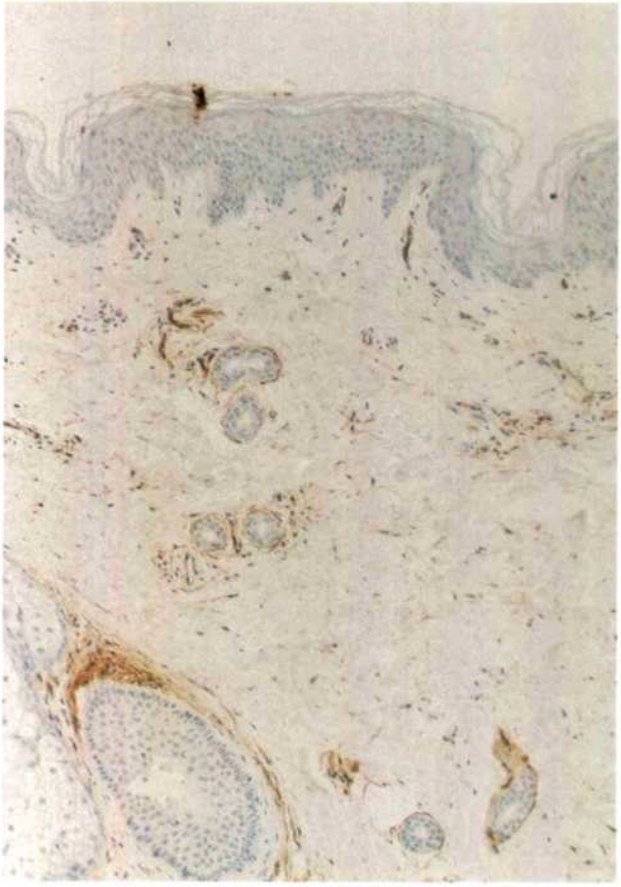

Figure 3. Immunoperoxidase staining of normal control skin for CD34 shows positive staining of endothelial cells, perifollicular and perisweat duct cells, and other stromal cells in the dermis (× 50).

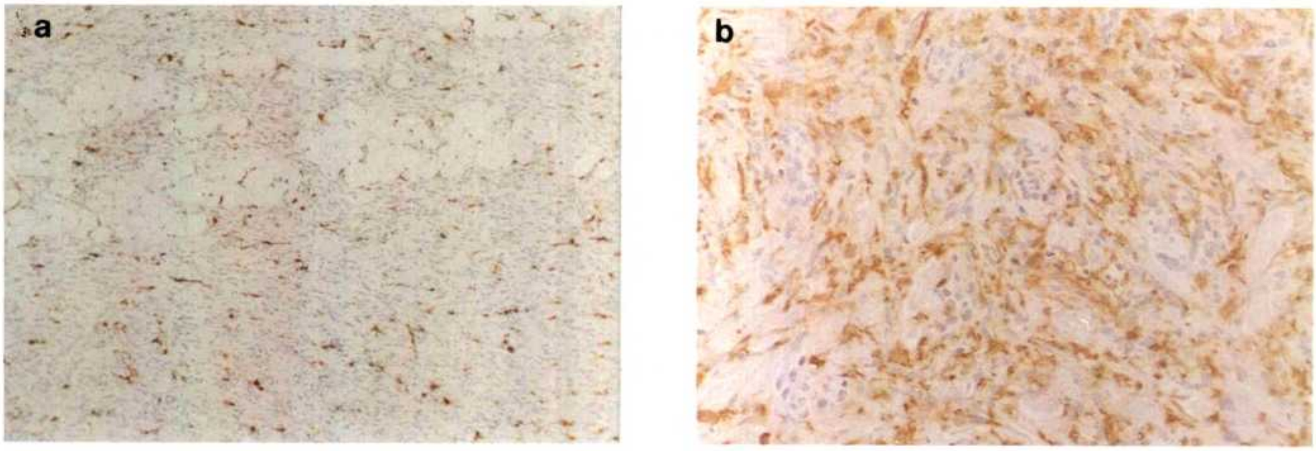

Figure 4. Immunoperoxidase staining of dermatofibrosarcoma protuberans and dermatofibroma for factor XIIIa. (a) Dermatofibrosarcoma protuberans: scattered dendritic cells amongst the tumour cells reveal factor XIIIa (× 200). (b) Dermatofibroma: most of the tumour cells reveal factor XIIIa (× 400).

haemosiderin deposits, epidermal hyperplasia, radial or vertical extension of tumour cells into the subcutaneous tissue predominantly along the septa, and smooth and bulging base of the tumour; DFSP—infrequent inflammatory infiltrate, thin epidermis, extension of tumour cells into the subcutaneous tissue in a multilayered pattern with bundles of spindle cells orientated parallel to the skin surface, or a honeycomb or lacelike pattern in which tumour cells extended along septa and between fat cells. Rehydrated sections (4-μm thick) were preincubated with trypsin, treated with 1% hydrogen peroxide in methanol for blocking endogenous peroxidase,[25] followed by an avidin–biotin peroxidase technique described previously.[26,27] The mouse reagents of the avidin–biotin peroxidase technique (Histofine SAB-PO(M) kit, Nichirei C, Tokyo, Japan) and the rabbit reagents (Histofine SAB-PO(R) kit) were utilized for CD34 and CD68 staining and for the human von Willebrand factor and the factor XIIIa staining, respectively. The sections were developed with diaminobenzidine solution and 1% hydrogen peroxide, and counterstained with Mayer's haematoxylin.

Results

We found a strong positive staining of tumour cells for CD34 antigen in all seven cases of DFSP (Table 1, Fig. 1a, b and c), and in one out of nine cases of MFH. CD34 antigen was observed on the cytoplasmic membrane of tumour cells, and partly on the fibrous tissue. In all cases of dermatofibroma, including the fibrous type extending into subcutaneous tissue, seven out of eight cases of MFH, two cases of infantile myofibromatosis, two cases of hypertophic scar, two cases of keloid, and one case of fibrosarcoma, the tumour cells were negatively stained with anti-CD34 antibody, while endothelial cells in the tumour tissue were CD34 positive (Fig. 2a and b). Only one case of MFH, whose clinical and histopathological features were distinct from DFSP, showed positive CD34 staining. In uninvolved tissues of all the specimens we examined, and normal control skin, CD34 was expressed on endothelial cells, dermal dendritic cells, and perifollicular cells (Fig. 3).

In contrast with CD34, factor XIIIa was not expressed on tumour cells in any of the cases of DFSP, although its staining pattern in DFSP was variable; in three out of the seven cases, factor XIIIa-positive dendritic cells were scattered among the tumour cells (Fig. 4a), whereas in the remaining four cases, no factor XIIIa-positive cells were present in the tumour tissue. In all the cases of dermatofibroma varying numbers of cells were positively stained with anti-factor XIIIa antibody (Fig. 4b). In four out of seven cases of MFH there were scattered factor XIIIa-positive dendritic cells, in two cases no factor XIIIa-positive cells were present in the tumour tissues, and in the remaining case most of the tumour cells with atypical nuclei were factor XIIIa positive. Von Willebrand factor expression was limited to endothelial cells in tumour tissues and uninvolved skin. No tumour cells in any of the fibrohistiocytic tumours examined stained with anti-CD68.

Discussion

In spite of reports of various histopathological characteristics and many immunohistochemical studies on DFSP,[1–7] there is no useful marker for DFSP which precisely differentiates it from other fibrohistiocytic tumours. Barr *et al.*[28] have reported that a stroma composed of only non-polarizable collagen differentiates DFSP from dermatofibroma and fibromatosis, which contain amounts of polarizable collagen. Kamino and Jacobson[8] have presented histological criteria to differentiate DFSP from dermatofibroma extending into the subcutaneous tissues, and include the content of polarizable and non-polarizable collagen in these criteria. While examining CD34 expression on various fibrohistiocytic tumours immunohistologically, we found that all the tumours diagnosed as DFSP according to these criteria express CD34, whereas other fibrohistiocytic tumours, such as dermatofibroma, malignant fibrous histiocytoma (with the exception of one case), and infantile myofibromatosis, are CD34 negative. These CD34 positive tumour cells of DFSP did not express any other specific markers, including von Willebrand factor or factor XIIIa. Therefore, CD34 staining can be an extremely useful diagnostic marker to differentiate DFSP from other fibrohistiocytic tumours. Cerio *et al.*[5] suggested DFSP could be distinguished from dermatofibroma by the expression of factor XIIIa on the tumour cells. They demonstrated that DFSP was factor XIIIa negative, whereas dermatofibroma was factor XIIIa positive. However, the present study shows that factor XIIIa is not absolutely negative in all the cells in DFSP. In some cases, intermingled dendritic cells may show positive staining. Thus, a combination of staining patterns for CD34 and factor XIIIa can make the diagnosis of DFSP more reliable. Indeed all of our cases of DFSP, diagnosed on the histological criteria of Kamino and Jacobson,[8] were CD34 positive and the tumour cells were factor XIIIa negative. Furthermore, as an added benefit, CD34 staining may be performed on formalin-fixed, paraffin-embedded tissue sections. Recently, several papers have reported the usefulness of Mohs' surgery for DFSP,[29,30] and staining with anti-CD34 antibody could be used to determine the excision margins.

These unique phenotypic characteristics of DFSP, i.e. the CD34 positive, von Willebrand factor negative, and factor XIIIa negative staining pattern, strongly suggest that DFSP originates from the CD34 positive dermal spindle-shaped cells. Traweek *et al.*[31] and Nickoloff[24] reported that vascular neoplasms, including Kaposi's sarcoma, are CD34-positive tumours. However, the tumour cells of vascular neoplasia are also factor XIIIa positive, while those of DFSP are factor XIIIa negative, indicating that these tumours originate from different dermal components. CD34 is also considered a good marker for microvascular endothelial cells.[32] The negative staining of DFSP with anti-von Willebrand factor antibody indicates that DFSP does not originate from microvascular endothelial cells. These observations indicate the presence of at least three different kinds of dermal mesenchymal cells other than endothelial cells in the skin, i.e. CD34(−) factor XIIIa(−) cells as seen in most of the fibroblasts in the reticular dermis, CD34(+) factor XIIIa(−) cells as seen in DFSP, and CD34(−) factor XIIIa(+) cells as seen in dermatofibroma. We are now continuing our investigations in order to elucidate the functional characteristics of these dermal cells.

Finally, in the present study, one out of nine cases of malignant fibrous histiocytoma was found to stain with CD34. Staining with anti-factor XIIIa revealed three staining patterns in MFH. In type 1 there were no factor XIIIa-positive cells in tumour tissues; in type 2 there were scattered factor XIIIa-positive dendritic cells in the tumour tissues, and in type 3 tumour cells themselves expressed factor XIIIa. These data suggest heterogeneity of malignant fibrous histiocytoma.

Acknowledgments

We wish to thank Dr T.Kasai, Head of the Department of Dermatology, Sendai National Hospital, and Dr O.Maie, Associate Professor, Department of Dermatology, Akita University School of Medicine, for providing several histopathological specimens.

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British Journal of Dermatology (1992) 127, 85–90.

Further studies on the actions of endothelin-1 on blood flow in human skin

C.B.BUNKER,† M.L.COULSON,* N.A.HAYES,* P.M.DOWD† AND J.C.FOREMAN*

*Departments of *Pharmacology and †Dermatology, University College and Middlesex School of Medicine, University College London, Gower Street, London WC1E 6BT, U.K.*

Accepted for publication 12 February 1992

Summary When injected into human skin, endothelin-1 produces intense vasoconstriction localized to the site of the injection, but this area of vasoconstriction is surrounded by vasodilatation which spreads several centimetres from the injection site. The vasodilatation induced by intradermal injection of endothelin-1 (63 pmol) into human skin is prevented by local anaesthetic. Pretreatment of human skin with capsaicin also inhibits this response. Pretreatment of subjects with the selective histamine H1-receptor antagonist cetirizine, 10 mg orally 4 h before intradermal injections, inhibited vasodilatation caused by the intradermal injection of histamine (750 pmol), endothelin-1 (63 pmol), and carbachol (750 pmol). Endothelin-1 (0·3–10 μM) and carbachol (1–30 μM) failed to induce histamine release from rat peritoneal mast cells. We conclude that the vasodilatation caused by intradermal injection of endothelin-1 into human skin is neurogenic and is probably mediated by neuropeptide-containing primary afferent neurones. Because neither carbachol nor endothelin-1 cause histamine release from mast cells, our data suggest that histamine release from mast cells at the effector end of the axon reflex is responsible for the carbachol- and endothelin-induced vasodilatation in human skin.

Endothelins (ETs) are a family of 21-amino acid peptides, endothelins-1, -2, and -3, which are coded for by three genes which coexist in the human genome.[1] Endothelins are vasoconstrictor peptides produced by endothelial cells[2] and have been shown to be potent vasoconstrictors in rabbit skin, reducing basal blood flow caused by the vasodilator peptide calcitonin gene-related peptide (CGRP).[3]

In human skin *in vivo*, ET-1, 1–100 pmol, has been shown to produce intense vasoconstriction localized to the site of intradermal injection, and also to reverse the increase in skin blood flow caused by CGRP.[4] In addition to vasoconstriction at the site of injection in human skin, ET-1 induces a flare, due to increased skin blood flow, which spreads for several centimetres around the injection site.[4] It has been postulated that this increase in blood flow is caused by an axon reflex response to ET-1.

Despite its vasoconstrictor activity, endothelin as a bolus injection *in vivo* causes a decrease in arterial blood pressure in rats,[5] cats[6] and dogs,[7] but intravenous infusion causes a rise in blood pressure in rats[8] and dogs.[9] The *in vivo* vasodilator activity of endothelins-1 and -3 in cats demonstrates cross-tachyphylaxis, is dependent upon the rate of administration, and may not be caused by the generation of endothelium-derived relaxing factor (EDRF).[10]

In this paper, we describe the flare produced by the intradermal injection of endothelin-1 in human skin and use pharmacological methods to analyse its mechanism. The effects of carbachol and endothelin-1 in human skin are used to draw conclusions about the mechanism of the axon reflex in human skin.

Methods

Human skin studies

Local Ethical Committee approval was obtained for the study. Adult volunteers of both sexes (age range 21–42 years), who had no significant past or current medical history, were used for the experiments. All gave informed consent. None of the subjects was receiving medication at the time of the study or for 4 weeks prior to the study.

Histamine acid phosphate (Sigma, Poole, U.K.), carbachol (BDH, Poole, U.K.), endothelin-1 (Novabiochem U.K. Ltd, Nottingham, U.K.), substance P (Peninsula Laboratories, Merseyside, U.K.), and calcitonin gene-related peptide (Peninsula Laboratories) were all diluted in sterile, endotoxin-free saline from neutralized stock

Correspondence: Dr J.C Foreman, Department of Pharmacology, University College London, Gower Street, London WCIE 6BT, U.K.

solutions (about 1 mM). Intradermal injections of 25 μl were placed in randomized sites in the skin of the volar surfaces of both forearms. Assessment of the responses was made blind. The flare response was measured in two ways: (i) the area of the visible flare was traced on to acetate film and the area calculated from the mass per unit area of the acetate film, and (ii) laser-Doppler flowmetry was used to measure blood flow in the skin before, and at various times after injections. Because laser-Doppler flowmetry does not provide an absolute measure of skin blood flow, the results, in arbitrary units, are expressed as the change in flux, produced after injection. The times at which measurements were made are given in the results. Infra-red ($\lambda = 780$ nm) and helium–neon ($\lambda = 633$ nm) lasers were used, but as there was no difference between the measurements made with the two lasers, only the data with the infra-red laser are given.[11] The laser-Doppler flowmeter (Moor Instruments, Oxford, U.K.) produced a power output at the probe of approximately 1 mW for both lasers.

In the experiments with capsaicin, skin was pretreated with capsaicin cream (2% w/v; Dermal Laboratories, U.K.) three times daily, under occlusion, for 4 days. In experiments with xylocaine (20 mg/ml), the local anaesthetic was mixed with endothelin-1 in the same injection volume. In experiments with an H1-receptor antagonist, subjects were given cetirizine (Zyrtec, UCB Pharma, Belgium) 10 mg, orally 4 h prior to the experiment.

Histamine release

Mixed peritoneal cells from Sprague–Dawley rats were prepared as previously described.[12] Aliquots of washed peritoneal cells were incubated with substance P, carbachol or endothelin-1 at the concentrations stated in the results, in a total volume of 1 ml for 10 min at 37°C. Cells were prewarmed to 37°C before addition of the peptide or carbachol. The composition of the incubation medium has previously been described in full[12] and experiments were performed both in the presence, and absence, of extracellular calcium, 1 mM. After incubation the cells were centrifuged into a pellet (1000 *g* for 5 min) and the supernatant assayed for released histamine. The pellets were disrupted by heating to 100°C for 5 min and the residual histamine from the pellets was assayed. Histamine was assayed fluorimetrically after alkaline condensation with *o*-phthalaldehyde[12] and histamine release was expressed as a percentage of total cell histamine. In any one experiment all samples were performed in duplicate. None of the compounds used caused interference with the histamine assay. Total histamine release upon addition of peptide or carbachol has been corrected by subtracting the histamine release in the absence of peptide or carbachol (spontaneous release), which was between 2 and 6% of total cell histamine.

Results

Rat peritoneal mast cells which released histamine in response to substance P (2 and 5 μM) failed to release histamine in response to endothelin-1 (0·3–10 μM) and carbachol (1–30 μM) (Fig. 1). The experiments were carried out in the absence of extracellular calcium. Similar responses occurred in the presence of extracellular calcium (1 mM) (data not shown).

Figure 2 shows the time course of the flare response to endothelin-1 in human skin. The response is still marked at 30 min after injection of the peptide intradermally. Xylocaine (20 mg/ml) alone did not produce a significant flare, and when mixed with endothelin-1, xylocaine abolished the flare to endothelin-1.

In skin pretreated with capsaicin, the visible flare and the associated increase in blood flow caused by both endothelin-1 and histamine (63 and 750 pmol/site, respectively) were almost completely inhibited (Fig. 3). The weal response to histamine was unaffected by capsaicin pretreatment. Endothelin-1 did not cause a weal in human skin.

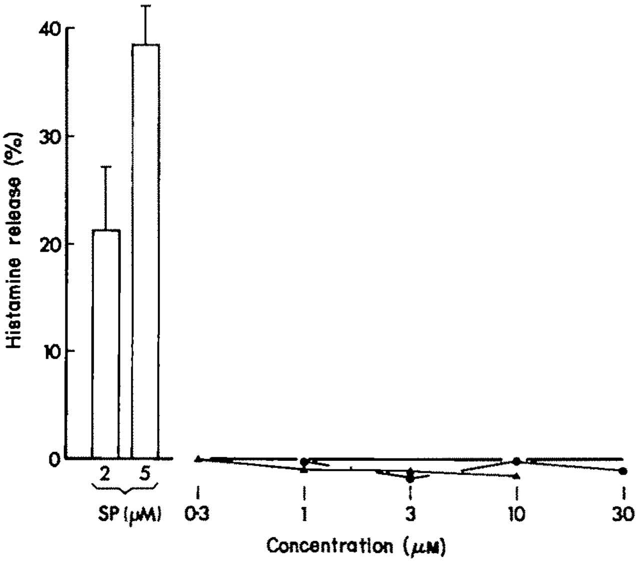

Figure 1. Action of substance P (open columns), carbachol (●—●) and endothelin-1 (▲—▲) on the release of histamine from rat peritoneal mast cells. Each data point is the mean from four separate experiments. Vertical bars indicate SEM but have been omitted from part of the data for clarity.

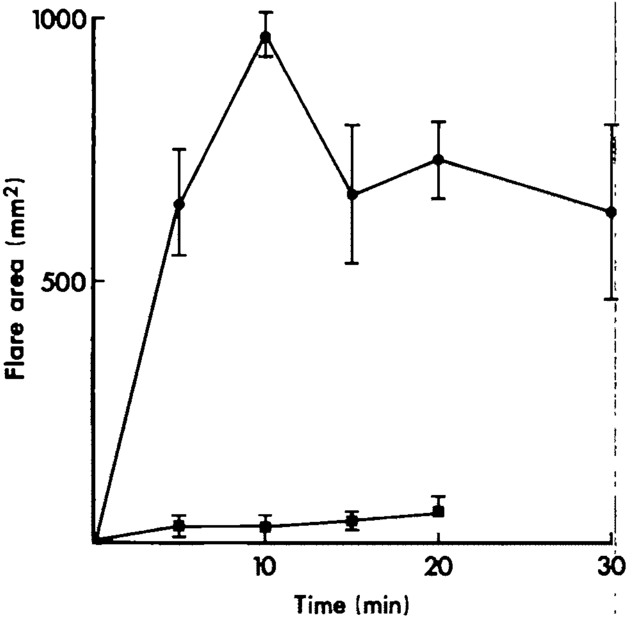

Figure 2. The effect of the local anaesthetic, xylocaine (20 mg/ml), on the area of the flare induced by endothelin-1 (63 pmol) in human skin. ●—●, endothelin-1 alone; ■—■, endothelin and xylocaine. Results are from 10 experiments and vertical bars indicate SEM.

Pretreatment of subjects with the H1-receptor antagonist cetirizine caused almost complete suppression of the visible flare (reduced by 95% at 2 min and 90% at 10 min) and of the increased blood flow caused by histamine (750 pmol/site) (reduced by 80% at 2 min and 78% at 10 min; Fig. 4). The flare and increased blood flow in response to endothelin-1 were similarly inhibited by the antihistamine but less so than the response to histamine. Flare caused by endothelin-1 was reduced by 76% at 2 min and 63% at 10 min, while the increased blood flow was reduced by 52% at 2 min and 46% at 10 min.

Figure 5 shows that the flare responses and increased blood flow induced by histamine (750 pmol/site) and carbachol (750 pmol/site) were also reduced by pretreatment of the subject with cetirizine. CGRP (25 pmol/site), a directly acting, non-neurogenic vasodilator in human skin,[13] was included as a control to demonstrate that cetirizine did not have a vasoconstrictor action at the dose employed in these studies. The responses to CGRP were measured 60 min after intradermal injection because the response to this peptide is slow to develop.[13]

Discussion

In human skin, intradermal injection of endothelin-1 produces vasoconstriction localized to the injection site, accompanied by an area of vasodilatation which spreads

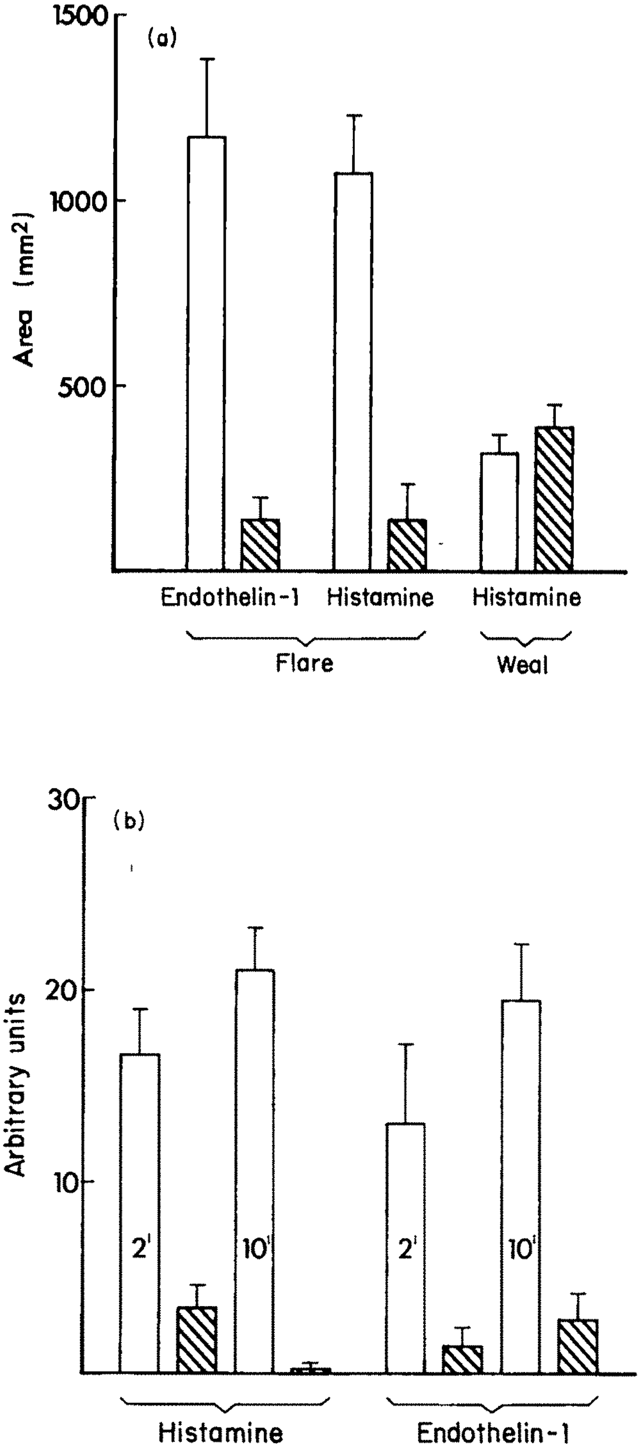

Figure 3. The effect of pretreatment of human skin with capsaicin on: (a) the area of the visible flare (measured at 2 min) and weal (measured at 15 min) induced by intradermal injection of histamine (750 pmol) or endothelin-1 (63 pmol). (b) The change in blood flow measured by laser-Doppler flowmetry at 2 and 10 min following intradermal injection of the same doses of histamine or endothelin-1. □, responses in untreated skin; ■, responses in capsaicin-treated skin. Each data point is the mean from four separate experiments and vertical bars show the SEM.

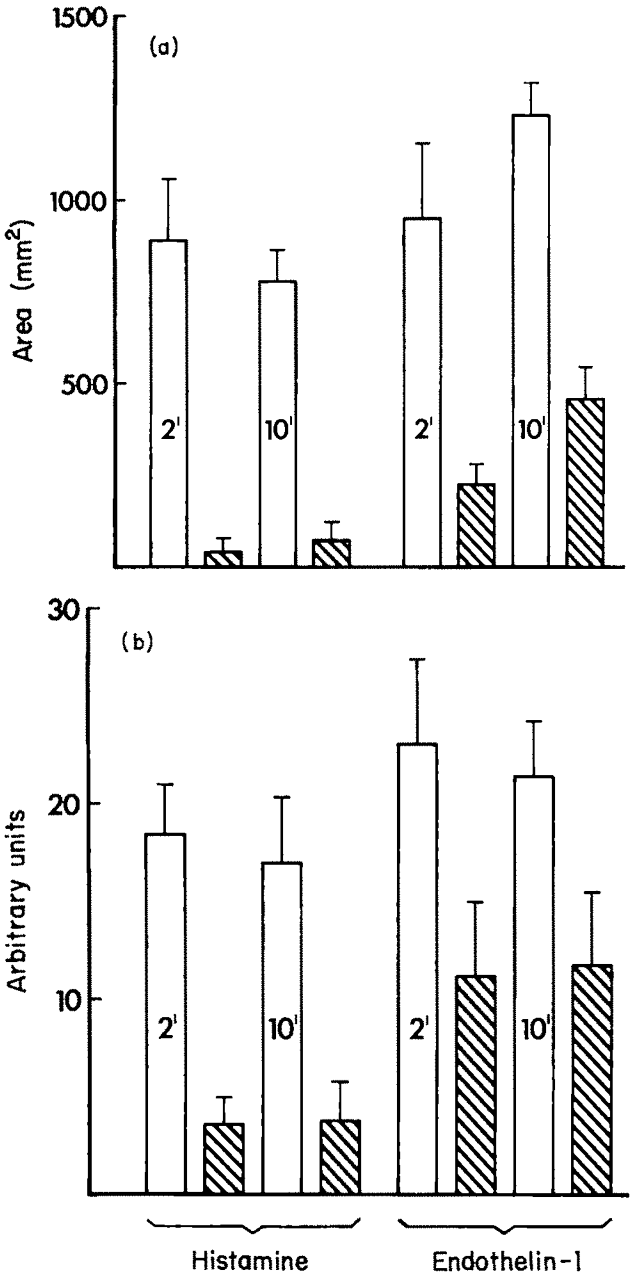

Figure 4. The effect of pretreatment of subjects with cetirizine on: (a) the area of the visible flare induced by intradermal injection of endothelin-1 (63 pmol) or histamine (750 pmol). (b) The change in blood flow measured by laser-Doppler flowmetry after intradermal injection of the same dose of histamine or endothelin. Measurements were made at 2 and 10 min after intradermal injections. □, responses before cetirizine treatment, ■, responses after cetirizine treatment. Each data point is the mean from five separate experiments and vertical bars show the SEM

several centimetres around the central area of vasoconstriction.[3,4] In this study we have shown that the vasodilatation induced by endothelin-1 is inhibited by local anaesthetic and by capsaicin pretreatment of the skin. Local anaesthetic blocks nerve fibres within the skin and it appears, therefore, that intact neuronal function is required for the vasodilator action of endothelin-1. Although unlikely, we cannot exclude the possibility that a chemical interaction between xylocaine and endothelin-1 could explain the data. More specifically, however, capsaicin has been used to desensitize primary afferent neurones in the skin and prevent them from functioning.[14] We conclude, therefore, that vasodilatation induced by endothelin-1 involves intact primary afferent neurones in the skin. The findings are consistent with the hypothesis that endothelin-1 induces a flare which is neurogenic in origin and therefore similar to the flare described by Lewis[15] following intradermal injection of histamine. The basis of the neurogenic mechanism of flare is the axon reflex[16] in a primary afferent C-fibre. It is suggested that endothelin-1 causes activation of the C-fibre at the site of injection, causing impulses to pass along the neurone. These impulses spread antidromically into the lateral, peripheral branches of the C-fibres and cause release of neuropeptides from the endings of these fibres, which are in close contact with mast cells near blood vessels.[17,18] The neuropeptides likely to be released by this process include substance P,[19] CGRP,[20] neurokinin A[21] and somatostatin.[22] It is considered most likely that substance P and CGRP are the mediators of neurogenic vasodilatation.[23]

Lewis[15] originally proposed that histamine is a mediator of neurogenic vasodilatation, and moreover, substance P releases histamine from mast cells.[12,24] An important question concerns the point in the axon reflex mechanism of neurogenic vasodilatation at which histamine release from mast cells occurs. A number of experimental observations are consistent with a role for histamine release from mast cells at the effector end of the axon reflex.[25] In other words, it is suggested that the antidromic impulses in the C-fibre branches cause the release of neuropeptides, which then act on mast cells to release histamine, which serves as the vasodilator substance. In contrast, other experimental observations[26] suggest that neuropeptides, not histamine, are the final mediators of vasodilatation. In this case, the role of histamine is considered to be as an activator of the C-fibre: histamine release from mast cells occurring at the point at which the stimulus to the axon reflex is applied. The data presented in this paper permit some clarification of the role of the mast cell and histamine release in the axon reflex.

Neither endothelin-1 nor carbachol caused histamine release from rat peritoneal mast cells, which have been

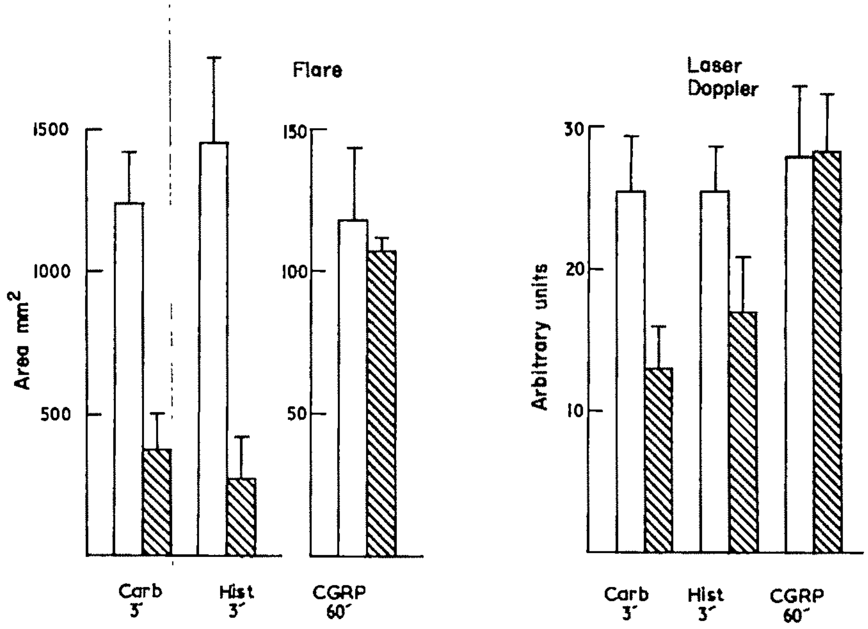

Figure 5. The effect of pretreatment of the subject with cetirizine on the flare area and change in blood flow in human skin following the intradermal injection of carbachol, 750 pmol/site, histamine, 750 pmol/site and CGRP, 25 pmol/site. □, before cetirizine; ■, responses after cetirizine pretreatment. The time at which measurements were made after intradermal injection are shown beneath the columns. The data are from five separate experiments and vertical bars show SEM.

shown to have similar properties to human skin mast cells in studies of histamine release.[12,27] Our observation of the lack of action of endothelin-1 on rat mast cells has been confirmed in human skin mast cells (M.K.Church, personal communication). We suggest, therefore, that intradermal injection of endothelin-1 or carbachol does not cause mast cell degranulation and histamine release at the injection site. Thus, it is unlikely that the activation of the axon reflex by these two agents in human skin is caused by histamine. However, our data clearly demonstrate that the neurogenic vasodilatation caused by intradermal injection of endothelin-1 and carbachol is markedly inhibited by the selective H1-receptor antagonist, cetirizine.[28] Moreover, we have shown that cetirizine does not block the non-neurogenic vasodilatation caused by CGRP, indicating that cetirizine is not a vasoconstrictor. These data are, therefore, consistent with histamine acting as the final vasodilator substance in axon reflex flare or vasodilatation caused by intradermal injection of endothelin-1 or carbachol. The same may be argued for platelet-activating factor (PAF) which does not release histamine[29] but causes a flare when injected intradermally into human skin. This PAF-induced flare is inhibited by H1-receptor antagonists,[30,31] and is blocked by depleting skin of its histamine by pretreatment with compound 48/80.[32]

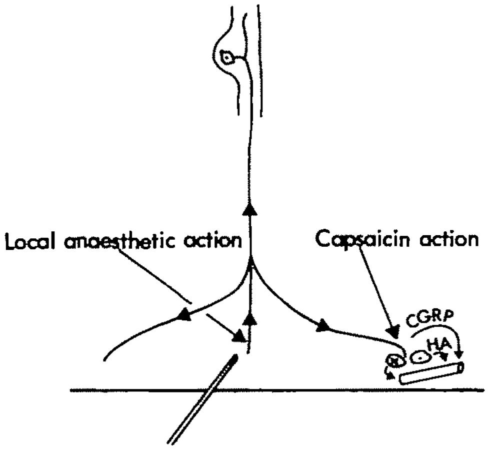

Figure 6. Model of the axon reflex in human skin to explain the data presented in this paper. Endothelin-1 and carbachol directly stimulate the C-fibre at the site of injection. Substance P release at the effector end of the axon reflex stimulates histamine (HA) release from mast cells. Local anaesthetic prevents the activation of the axon reflex by ET-1. Capsaicin, by depleting nerves of their neuropeptide content prevents axon reflex vasodilatation induced by ET-1. Cetirizine blocks the action of histamine on blood vessels and partly inhibits axon reflex vasodilatation by ET-1 but does not prevent vasodilatation caused by direct action of released neuropeptides (e.g. CGRP) on the vessels.

We propose, therefore, the model shown in Figure 6. Endothelin-1, carbachol or PAF activate the C-fibre by a mechanism independent of histamine release. Impulses arriving at the effector end of the axon reflex release neuropeptides which, in turn, cause histamine release from mast cells. The histamine then produces vasodilatation which is observed as a flare. There is good evidence that neurones form close associations with mast cells so that this process may be achieved.[17,18] Our data show that an H1-receptor antagonist inhibits the response to endothelin-1 less than it inhibits the response to histamine itself, and this may imply that neuropeptides such as CGRP may act directly on the vessels to cause vasodilatation, in addition to the action of histamine released from mast cells by neuropeptides.

The problem remains that not all the published data are consistent with the model proposed in Figure 6.[26] For some forms of stimulation of the axon reflex, mast cells and histamine release appear to be involved in the stimulus, and there is little evidence for mast cell involvement at the effector end of the reflex under these conditions. At present this cannot be explained, but we speculate that whether or not the mast cell has a role at the effector end of the axon reflex may depend on the nature of the stimulus, or on the particular C-fibres recruited. Such an hypothesis requires testing but the data presented in this paper are, we believe, clearly consistent with a role for mast cells at the effector end of the axon reflex when endothelin-1, carbachol or PAF are the stimuli.

Acknowledgments

We are grateful to UCB Pharma, Braine l'Alleud, Belgium for generous financial support. We thank our colleagues who so willingly gave their time to volunteer for this study.

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British Journal of Dermatology (1992) 127, 91–96.

Quantification of *n*-alkanes in stratum corneum in the hereditary ichthyoses

M.R.JUDGE, N.MORSE-FISHER,* M.MANKU* AND J.I.HARPER
Department of Dermatology, The Hospitals for Sick Children, Great Ormond Street, London, U.K.
**Efamol Research Institute, Kentville, Nova Scotia, Canada*

Accepted for publication 1 April 1992

Summary

Chromatographic assay of *n*-alkanes in skin showed detectable levels in normal controls and in patients with various forms of hereditary ichthyosis. Raised *n*-alkanes were found in some, but not all, patients with non-bullous and bullous ichthyosiform erythroderma and in individual patients with lamellar ichthyosis, ichthyosis vulgaris and Netherton's syndrome.

The finding of elevated scale *n*-alkanes is neither consistent in ichthyosis, nor specific to any one type of ichthyosis, and *n*-alkane assay is not helpful in distinguishing one type of hereditary ichthyosis from another. The source of *n*-alkanes in ichthyotic scale and their role, if any, in the pathogenesis of ichthyosis remain obscure.

Human stratum corneum is composed of protein-rich anucleate keratinocytes and a lipid-rich intercellular matrix, which is continually undergoing biochemical change. Extractable lipids comprise 10–20% of stratum corneum weight and are mainly composed of non-polar lipid classes such as ceramides, sterols, free fatty acids and triglycerides, with small amounts of polar phospholipids and cholesterol sulphate present in the lower layers. Alkanes have been found to constitute 0·5–5% of normal stratum corneum lipids.[1,2,3]

Alkanes are non-polar hydrocarbons, derivatives of petroleum compounds, which are present in small amounts in the atmosphere and in large quantities in paraffin-containing emollients. The alkanes identified in insect and other animal waxes are predominantly straight-chain and are given the prefix '*n*', whereas plant waxes also contain branched alkanes. Petrogenic *n*-alkanes are composed of almost equal amounts of odd and even carbon chain lengths.[4]

Williams *et al.*[1] have cited the finding of elevated levels of *n*-alkanes, up to 25%, in the skin scale of patients with non-bullous ichthyosiform erythroderma (NBIE) and in some patients with bullous ichthyosiform erythroderma (BIE) and psoriasis, and suggested that they might be responsible for the hyperproliferation characteristic of these disorders. In order to assess the consistency and specificity of a raised *n*-alkane level in certain forms of hereditary ichthyosis, we developed an *n*-alkane assay using thin-layer and gas chromatography and applied it to the study of scale lipid samples from a variety of inherited ichthyoses.

Methods

Collection of lipid samples

Sixteen patients, nine adults and seven children (under 16 years), age range 18 months–67 years, suffering from various forms of hereditary ichthyosis were included in the study. Five had non-bullous ichthyosiform erythroderma (NBIE), three bullous ichthyosiform erythroderma (BIE), three X-linked ichthyosis (XLI), two Sjögren–Larsson syndrome (SLS) and one each had lamellar ichthyosis (LI), ichthyosis vulgaris (IV) and Netherton's syndrome. Patients were instructed to avoid all emollients, especially on the legs, for a minimum of 3 weeks before testing. Controls included: three subjects without any history of the skin disease, who had plaster of Paris occlusion of the tested site for 6 weeks; a sample of normal plantar shavings; a sample of plantar scale from a patient with localized palmoplantar keratoderma (PPK).

Scale samples were collected, using a no-touch technique, from the lower legs, apart from the two control plantar skin samples. Scale samples were stored at −70°C in chromic acid-washed glassware.

In order to assess the contribution of *n*-alkanes from topically applied emollients, five patients (two with NBIE and three with BIE) who had been applying topical emollients containing paraffins up to the time of sampling, were also tested. In addition, emollients

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Correspondence: Dr J.I.Harper, Consultant Dermatologist, The Hospitals for Sick Children, Great Ormond Street, London WC1N 3JH, U.K.

commonly used in the U.K. were analysed for their alkane content.

Processing of samples

All glassware was solvent-cleaned to exclude lipid contamination. Scale lipid was extracted with 10 ml of chloroform : methanol, 2 : 1, in a 1-h incubation, at room temperature. The solvent layer was filtered through solvent-washed glass wool and evaporated under a nitrogen stream. Lipid mass and its percentage of scale weight was calculated and it was redissolved in 500 μl of solvent.

A sample of either 100 or 200 μl of lipid extract of known concentration was spotted on to a 20 × 20 cm silica 60 thin-layer chromatography (TLC) plate, 1·5 cm from the origin. Two samples were applied to each plate and an *n*-alkane control was included in each TLC run. The plates were developed in a solvent tank containing petroleum ether : diethyl ether : acetic acid, 80 : 20 : 1, and allowed to run to 10 cm. They were air-dried and run full length in a second solvent tank containing petroleum ether alone. The plates were air-dried and the *n*-alkane band, which was visible as a white band at the solvent front on the 'naked' plate, was enhanced by viewing under ultraviolet light. In addition the *n*-alkane band was identified by comparison with the standard and it was scraped and transferred to a test tube. The scrapings were mixed with 5 ml of hexane and 2 ml of water and, after centrifugation, the hexane layer was aspirated and evaporated to dryness under nitrogen. The resulting *n*-alkane extract was redissolved in 100–500 μl of hexane.

Each *n*-alkane sample was quantified by gas chromatography (GC) using a Hewlett–Packard 5890 series 2 gas chromatograph. The identities of individual *n*-alkane peaks were established by co-chromatography against known standards of specific carbon-chain length. The GC 30 m, 0·25 mm ID column contained fused-silica of 0·25 μm dF (Supelcowax 10). The helium carrier flow rate was 1 ml/min through the column and the oven temperature programme commenced at 130°C, rising by 2·5°C per minute to a final temperature of 230°C after 25 min. The injector temperature was 200°C and detector temperature was 220°C. Flow rate through the flame-ionization detector (FID) comprised helium 30 ml/min, hydrogen 30 ml/min and air 400 ml/min. The GC was equipped with a HP7673A automatic injector, a HP3365 chemical station and HP think-jet printer. Three microlitres of each sample were injected.

To calculate the *n*-alkane content per mg of skin scale-lipid extract, the mass of each peak, representing a specific *n*-alkane of known carbon-chain length and concentration, was calculated individually and all peaks were totalled. The mass of each individual *n*-alkane peak was assessed by comparison with the mass of the standard *n*-alkane of equivalent carbon-chain length using the following formula:

Mass of specific *n*-alkane (μg)

$$= \frac{\text{Mass of standard } (\mu\text{g}) \times \text{Area of sample peak}}{\text{Area of standard peak}}$$

$$\times \frac{\text{Total volume of alkane extract } (\mu\text{l})}{\text{Total volume of sample injected } (\mu\text{l})}$$

$$\times \frac{\text{Total volume of extract } (\mu\text{l})}{\text{Total volume of sample plated } (\mu\text{l})}$$

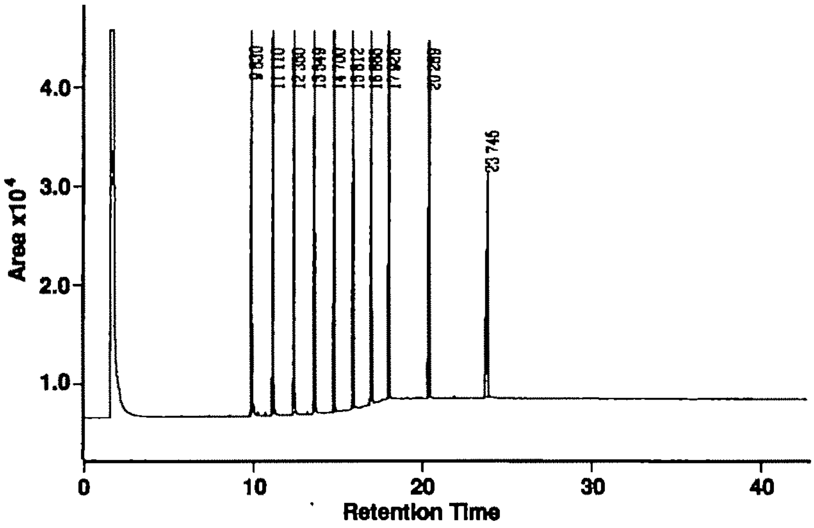

Figure 1. Gas chromatogram of composite *n*-alkane standard, showing individual peaks for known amounts of *n*-alkanes of carbon chain length C 19–26, 28 and 30.

Standards were available for *n*-alkanes of carbon-chain lengths C19–26 and C28 and C30 (the most prominent *n*-alkanes in animal tissues) and a composite *n*-alkane control (standard) was run after every four patient samples (Fig. 1). Peaks for *n*-alkanes of C18, C27, C29 and C31–33 chain lengths were calculated by extrapolation from the adjacent standard *n*-alkane peaks and this calculation may introduce a minor degree of error, which applies equally to all samples, to the overall results.

Serial dilutions, from 0·01 to 1·0 μg/μl, of the stock standard solutions of all the control *n*-alkanes were prepared and a 3 μl sample of each was injected using the same GC programme as for the patient samples. The GC recordings for each individual *n*-alkane standard produced completely linear graphs and these were used to calculate the amount of each individual *n*-alkane in patient samples.

Recovery of individual *n*-alkanes in a composite standard from TLC plates was approximately 10%. Duplicate samples yielded consistent alkane recoveries in all groups of ichthyosis and results were valid for intergroup comparison. In order to exclude environmental contamination, blank TLC plates were processed in a similar way with an internal standard, scraped and extracted. GC analysis detected no *n*-alkanes in the appropriate extracted 'band'. TLC plates precleaned with ethyl acetate showed no advantage in separation or yield and were not used.

Results

The *n*-alkane content in μg/mg of skin scale lipid extract in each ichthyosis patient and in the controls is shown in Table 1.

Four control scale samples contained 2·39–7·98 μg of *n*-alkane/mg of scale lipid with a ratio of odd to even carbon chain lengths of 0·85–1·01. There was no site-related difference between plantar (C3 and C4) and lower leg scale (C1 and C2) samples. In addition, the plantar shavings from the patient with PPK had an *n*-alkane level within this normal range.

Some of the scale samples from patients with ichthyosis had clearly raised levels of *n*-alkanes: three of the five with NBIE, one of the three with BIE and each of the individual patients with IV, LI and Netherton's syndrome had an *n*-alkane level >20 μg/mg. Only one of the three samples of XLI scale had a marginally raised value of 10·94 μg/mg. The other ichthyotic patients tested had levels within the normal range.

Table 1. *N*-alkane content of scale lipids from patients with ichthyosis and normal controls

Subject no.	Diagnosis	Age	Sex	Site*	Alkane value (μg/mg lipid)
1	NBIE	14	M	LL*	53·42
2	NBIE	17	M	LL	1·96
3	NBIE	20	M	LL	31·78
4	NBIE	68	M	LL	35·50
5	NBIE	6	F	LL	5·84
6	BIE	7	M	LL	2·65
7	BIE	2	M	LL	53·90
8	BIE	44	F	LL	3·46
9	XLI	10	M	LL	7·60
10	XLI	18	M	LL	4·20
11	XLI	30	M	LL	10·94
12	IV	27	F	LL	24·25
13	LI	6	F	LL	50·54
14	SLS	13	F	LL	6·68
15	SLS	10	F	LL	7·73
16	Netherton's syndrome	23	F	LL	20·27
C1†	PPK	24	F	P	3·57
C2	Normal	3	F	LL	2·39
C3	Normal	13	M	LL	7·98
C4	Normal	15	M	LL	3·60
C5	Normal	33	F	P	4·88

	Mean *n*-alkane value (μg/mg lipid)	Standard deviation
Normal (*n*=4)	4·71	±2·40
NBIE (*n*=5)	25·70	±21·56 (*P*= <0·05)
BIE (*n*=3)	20·00	±29·36 (*P*= >0·05)
XLI (*n*=3)	7·58	±3·37 (*P*= >0·05)

* LL, lower leg; P, plantar.

† C1–5, control scale samples.

Regarding the ratio of odd to even carbon chain length *n*-alkanes, the NBIE samples averaged 0·98, a ratio similar to that of the normal samples which averaged 0·94. The ratios in BIE, SLS and in particular XLI samples (respective averages 1·02, 1·08 and 1·17) are higher, indicating an increasing predominance of odd chain *n*-alkanes in these disorders.

A gas chromatogram of the *n*-alkanes in a sample of scale from a patient with NBIE shows a mixture of *n*-alkanes of varying chain lengths from C19–33 and roughly equal proportions of odd and even chain *n*-alkanes (Fig. 2). A chromatogram of a normal control scale sample is also shown (Fig. 3).

Of the five patients who had continued to apply emollients to their skin before sampling (Table 2), one had a markedly elevated *n*-alkane level, three had

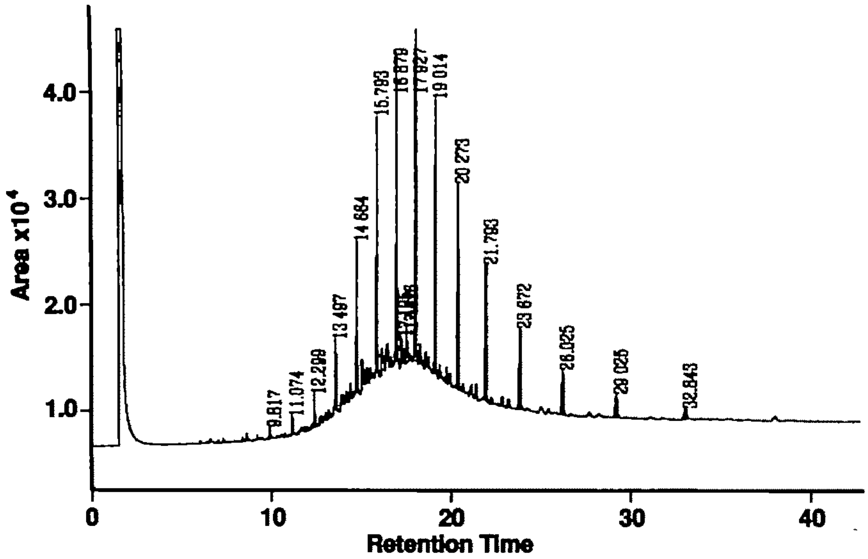

Figure 2. Gas chromatogram of *n*-alkane extract of scale lipid from a patient with NBIE (Subject No. 1, Table 1).

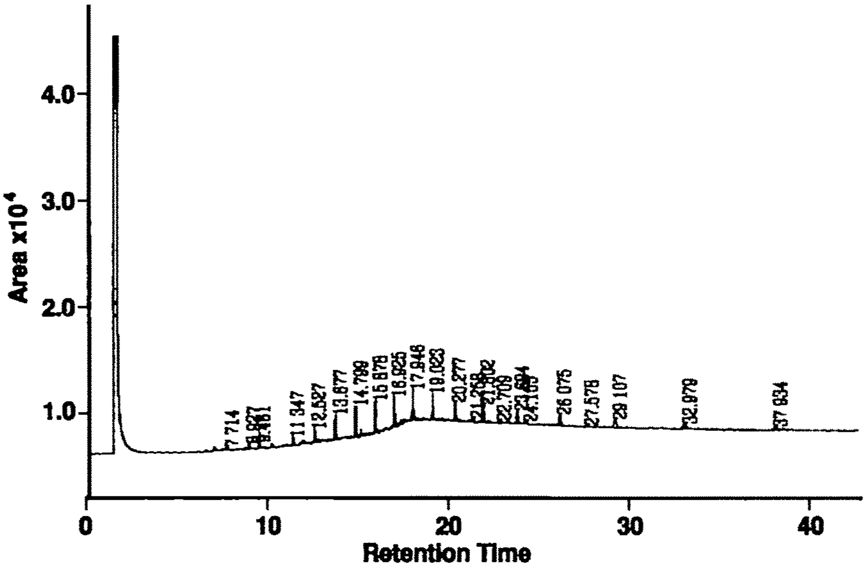

Figure 3. Gas chromatogram of *n*-alkane extract of scale lipid from a control normal subject (Subject No. C2, Table 1).

Table 2. Alkane levels in scale samples from patients with ichthyosis who were treated with emollients

Subject no.	Diagnosis	Alkane value (μg/mg lipid)
17	NBIE	9·44
18	NBIE	38·78
19	BIE	79·47
20	BIE	25·77
21	BIE	13·74

Table 3. Alkane concentrations of commonly used emollients and the corresponding odd to even carbon chain ratio

Product	Alkane content (μg/mg of product)	Odd:even ratio
Arachis oil	0·103	1·51
Alpha Keri*	1·618	0·70
Diprobase*	1·894	1 25
Unguentum Merck*	1·900	1·27
Aqueous cream	2·393	1·24
Oilatum*	10·772	1·55
Emulsifying ointment	12·950	1·12

significantly raised levels and one was slightly above the normal range. Except for Subject 19, the application of paraffin-containing emollients did not appear to influence scale *n*-alkane content. It would be important to obtain more detailed information on the nature, quantity and timing of treatment and, if possible, the alkane level in untreated scale from these patients.

Table 3 shows the results of the analysis of the individual emollients commonly used by patients with dry and scaly skin, and in general the *n*-alkane content reflects the amount of paraffin in each. Emulsifying ointment contains 70% paraffin and has the highest *n*-alkane level while aqueous cream and Diprobase® each contain 21% paraffin and have correspondingly lower levels.

Discussion

n-Alkanes are an important constituent of insect and plant cuticular lipids and contribute significantly to the structure and barrier function of their waxy integument.[6,7] Plant *n*-alkanes are generally restricted to carbon chain lengths between C25 and 35, with a marked predominance of odd chains,[6] whereas animal waxes and petroleum compounds contain a broader range with an equal distribution of odd and even chain lengths.[8] *n*-Alkanes are also widely distributed in the atmosphere and environment and are present in many food additives and wrappings, medications, mineral oils and paraffin waxes.

Lower levels of *n*-alkanes are found in mammalian epidermis and they may, in conjunction with ceramides, contribute to barrier function. However, there is no known *n*-alkane biosynthetic capability in mammalian skin,[9] although they can be catabolized by the cytochrome P450 system.[4]

It has been claimed that the *n*-alkanes detected in human skin, either skin surface lipids or scale extracts, result from environmental contamination. ^{14}C carbon-dating of normal human skin *n*-alkanes indicated a theoretical age of 30,000 years, implying that they could not have been synthesized by that tissue.[2] The variety of alkane carbon-chain profiles resembled those of petroleum oils and their virtual absence in cerumen further suggested that exogenous sources of alkanes were important.[1]

On the other hand, Lampe *et al.*[10] reported that *n*-alkanes were present at all skin sites regardless of exposure to the environment, in skin untreated with topical agents for weeks beforehand, and in spite of meticulous exclusion of potential contaminants in the laboratory. In addition, they have been identified in the stratum granulosum.[11]

Williams *et al.*[1] found large quantities of *n*-alkanes in untreated scale of certain patients with hereditary ichthyosis, in particular those with NBIE. They proposed that they were generated in the epidermis and excluded a dietary origin on the basis of normal *n*-alkane levels in red cells, serum and dermis of patients with NBIE. In NBIE scale there was a reciprocal reduction in triglycerides and free fatty acids, suggesting that fatty acids could be precursors of *n*-alkanes. They believed that the raised *n*-alkane levels in NBIE scale would help to differentiate it from the closely related disorder, LI, where levels were only slightly above normal. In a subsequent study, a small number of patients with BIE and psoriasis also had raised values, but prior application of emollients could have been responsible in some.[12] They postulated that the increased *n*-alkanes in NBIE epidermis might affect the liquid-crystalline phase-transition properties of stratum corneum lipids, resulting in a hardening of the bilipid layers at physiological temperatures and an increase in stratum corneum cohesion and brittleness. Topical application of *n*-alkanes to normal skin has been shown to induce epidermal hyperplasia,[13] scaling, mild erythema and a twofold increase in the epidermal labelling index in hairless mice.[14] However, we observed that elevated *n*-alkanes were not always associated with erythema.

In our study, scale derived from essentially normal skin yielded detectable though consistently low levels of *n*-alkanes. They showed a bell-shaped distribution of carbon chain lengths between C16–34 with both odd and even chain lengths. The odd-to-even chain ratio averaged less than or equal to one.

All patients with ichthyosis had detectable levels of *n*-alkanes (Table 1). Two patients with Sjögren–Larsson syndrome and two with X-linked ichthyosis had levels within the normal range, but with a predominance of odd chain alkanes. One patient each with Netherton's syndrome, ichthyosis vulgaris and BIE showed significantly raised alkane levels, while two with BIE had normal values. The single patient with LI also had a markedly elevated level (alkanes 50·5 μg/mg lipid). The highest group level occurred in NBIE (average 25·7 μg/mg lipid for five patients). However, two of the five patients with typical severe NBIE had low levels, well within the range for the normal scale samples. So whilst there was a definite trend towards raised *n*-alkanes in skin scale from patients with some forms of hereditary ichthyosis, this finding was neither reliably sensitive for nor specific to NBIE. Likewise, it did not help to differentiate NBIE from the one case of LI. Fortunately, there is little difficulty distinguishing NBIE from BIE clinically.

The wide range of carbon chain lengths from C18 to 34 in most patients and controls was also noted by Bortz *et al.*[2] who believed it implied environmental contamination. Our patients with NBIE and BIE who had treated their skin with paraffin-containing emollients also had variable scale *n*-alkane levels.

The relevance of raised epidermal *n*-alkanes in the pathogenesis of ichthyosis is obscure. They may aggravate erythema, hyperproliferation and scaling but, on the evidence of this study, their presence is not a *'sine qua non'* of erythrodermic ichthyosis, and a primary role in pathogenesis seems unlikely. Our results confirm that alkanes are consistently found in normal human skin scale and that values are confined to a narrow range. They do not help to differentiate exogenous contamination from possible epidermal synthesis. Skin scale *n*-alkane assay is not sufficiently reliable to be used as a diagnostic test for specific hereditary ichthyoses.

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British Journal of Dermatology (1992) **127**, 97–102.

Measurement of phosphoinositide-specific phospholipase C activity in mononuclear leucocytes from atopic and normal subjects

R.B.MALLETT, S.MYINT AND C.A.HOLDEN
Department of Dermatology, St Helier Hospital, Carshalton, Surrey SM5 1AA, U.K.

Accepted for publication 9 April 1992

Summary The intracellular inositol pathway is an important route for cell activation and relies on the stimulation of membrane-bound phosphoinositide-specific phospholipase C (PLC). Previously we have shown abnormalities of inositol metabolism in mononuclear cells (MNL) in atopic dermatitis (AD) using an indirect method. We now describe a direct method of measuring PLC activity in membrane and cytosol preparations of MNL in AD. We compare PLC activity in AD with that in normal controls and examine the effect of substrate concentration and nucleotide stimulation on the system. Our findings show increased membrane-bound PLC activity in AD compared with normal controls. Non-specific stimulation of AD PLC activity by nucleotides suggests that the enzyme of atopics is more sensitive to substrate-driven activity than that of non-atopics.

The search for the underlying abnormality in atopic dermatitis (AD) continues.[1] Szentivanyi initially proposed that the cellular hyporesponsiveness observed in atopy was related to partial β-adrenergic blockade.[2,3] Subsequent studies identified not only leucocyte cyclic AMP (cAMP) hyporesponsiveness to β-adrenergic agonists, but also to histamine, isoprenaline and PGE_1.[4,5] The latter findings implied a defect not limited to the β-adrenergic receptor, but occurring further down the cAMP pathway.

Hanifin's group proposed that an abnormality of an intracellular secondary messenger enzyme may be the central defect, and subsequently demonstrated elevated cAMP phosphodiesterase (PDE) levels in leucocytes from adults with AD, suggesting that the resultant increased breakdown of cAMP was a possible mechanism for the observed cAMP hyporesponsiveness.[6,7] This was an attractive explanation and correlated well with several immunological abnormalities found in AD, including increased basophil histamine release[8] and increased IgE synthesis.[9]

However, rather than a primary defect, elevated cAMP–PDE levels could be secondary to abnormalities of another messenger system, in particular the more recently described inositol pathway.[10,11] Activation of the inositol pathway (Fig. 1) relies on receptor stimulation of phosphoinositide-specific phospholipase C (PLC) to cleave membrane-bound phosphatidyl inositol

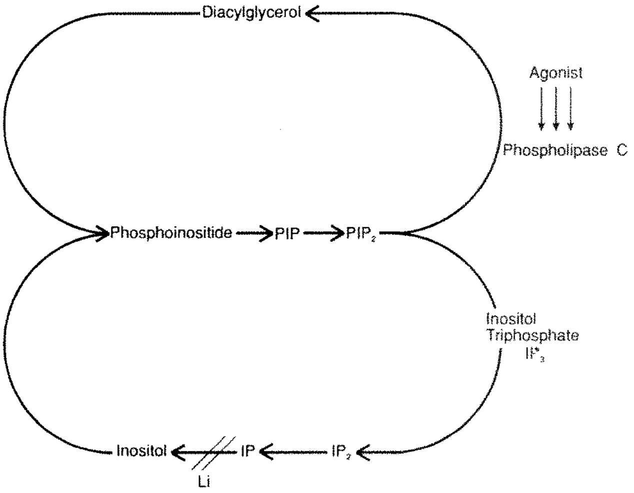

Figure 1. A simplified representation of the inositol cycle. Phosphotidyl inositol biphosphate (PIP_2) is the principal substrate for phospholipase C which cleaves PIP_2 into diacylglycerol (DAG) and inositol triphosphate (IP_3). Membrane bound DAG can activate protein kinase C to phosphorylate and activate effector enzymes. Within the cytoplasm IP_3 and its metabolites IP_2 and IP can mobilize calcium to cause enzyme activation. IP is dephosphorylated to inositol, a step inhibited by lithium.

Correspondence: Dr R.B.Mallett, Department of Dermatology, St Helier Hospital, Wrythe Lane, Carshalton, Surrey SM5 1AA, U.K.

4,5,bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5,triphosphate (IP_3). DAG then activates membrane-bound protein kinase C (PKC) which in turn activates cAMP-PDE. IP_3 causes mobilization of intracellular calcium and consequent cell activation.

The inositol pathway is an important route for cell activation via signal transduction of many hormones, neurotransmitters and inflammatory mediators. Recent immunological investigations in AD have focused on an apparent imbalance between interleukin 4 and γ interferon.[12,13] The mechanism of action of these cytokines is still to be fully elucidated. There is evidence to suggest that PKC activation via the glycosylphosphatidylinositol pathway may be important.[14,15] It is also possible that the classical inositol pathway may play a role. For these reasons we thought it important to investigate the inositol pathway in circulating mononuclear leucocytes (MNL) in patients with AD.

Our previous study showed the inositol pathway to be hyporesponsive to Con A stimulation in AD compared with normals. This employed an assay of inositol uptake by whole cells.[16] In order to try to analyse the separate stages of the pathway and to measure membrane PLC activity in MNL directly we have modified the method of Bergers et al. for measuring PLC activity in particulate preparations of rat brain.[17] We describe the method, compare results from patients with AD and non-atopic controls, and examine the effect of nucleotide stimulation on PLC activity.

Methods

Before commencing this study, hospital ethical committee approval was granted, and all subjects gave informed consent.

Subjects

Venous blood samples were taken between 8 and 10 a.m. from 14 patients with atopic dermatitis (AD) and 13 normal controls, who had avoided caffeine and methylxanthine-containing beverages and food for 12 h previously. The AD patients were young adults, fulfilling the criteria of Hanifin and Rajka,[18] with chronic disease receiving no systemic therapy, but controlled with topical medication only. Control subjects were young adult volunteers with no personal or family history of atopy who were prick-test negative to a battery of nine common allergens.

Mononuclear leucocyte separation

MNL were separated from heparinized venous blood by Ficoll–Hypaque centrifugation. The heparinized blood was diluted with an equal volume of phosphate-buffered saline containing 0·3 mM EDTA (PBS–EDTA) at 4°C, and carefully laid on to 3 ml Ficoll–Hypaque separation medium (Flow Labs, Rickmansworth, Herts, U.K,) in a 13-ml sterile plastic centrifuge tube (Sterilin Ltd, Feltham, U.K.) The tubes were then centrifuged at 400 *g* for 30 min. MNL were removed from the plasma/Ficoll–Hypaque interface by gentle suction. The isolated MNL were diluted 1:3 with PBS–EDTA and centrifuged at 400 *g* for 10 min. The supernatant was decanted and the MNL washed by resuspension in PBS–EDTA followed by centrifugation at 300 *g* for 10 min. The process was repeated twice at 250 *g* for 10 min to reduce platelet numbers. The MNL were finally resuspended at 20×10^6 cells/ml in buffer containing 20 mM Tris, 1 mM $CaCl_2$, 100 mM KCl, at pH 7·4. The MNL comprised 80–85% lymphocytes and 15–20% monocytes with less than 1% neutrophil contamination and viability greater than 95% by trypan blue exclusion.

Preparation of isolated membrane suspensions and cytosol

MNL were frozen and thawed × 3 in liquid nitrogen and then homogenized by 40 strokes in a glass Dounce homogenizer. The homogenate was centrifuged for 10 min at 250 *g* at 4°C and the supernatant aspirated and sonicated at 30-s intervals at 4°C for a total of 3 min in an Ultrasonic Rapidis 180 sonicator. The membranes were then centrifuged at 45,000 *g* for 20 min at 4°C in a fixed-head rotor in a Sorval RC 5-B. The supernatant was aspirated and the membrane pellet was finally resuspended in 1 ml buffer containing 20 mM Tris, 1 mM $CaCl_2$, 100 mM KCl, at pH 7·4, and stored at −70°C. Cytosol was obtained by collecting the supernatant after the 45,000 *g* centrifugation.

Phospholipase C assay

The assay relies on the conversion of lipid-soluble radiolabelled PIP_2 ($[^3H]$-PIP_2 (specific activity 1–5 Ci/mmol) Amersham International, Amersham, U.K.) by phospholipase C into DAG and water-soluble radiolabelled IP_3 (Fig. 1). Separation of the aqueous phase by chloroform/methanol extraction and measurement of its radioactivity count enables determination of PLC activity expressed in pmol PIP_2 hydrolysed/min/10^6 cells.

All glassware was siliconized to avoid loss of PIP_2 by surface absorption. The substrate was prepared from PIP_2 stocks (hot and cold) which were stored in chloroform/methanol (1:1). Cetrimide (Sigma Chemical Co Ltd, Dorset, U.K.) was stored, as a solution in ethanol. Immediately prior to assay appropriate volumes of the stock solutions were mixed to give a 'cocktail solution' containing 50 nmol/ml (total) PIP_2+0·5 µCi/mi [^{3}H]-PIP_2 and 0·4 mg/ml cetrimide. The 'cocktail' was air-dried to remove solvent and then an equal volume of buffer containing 20 mM Tris, 1 mM $CaCl_2$, 100 mM KCl, 25 mM LiCl, 1mM $MgCl_2$, at pH 6·8, was added and the preparation ultrasonicated for 30 s at 22–30°C using a ME 4·6 Metter Electronics Cavitator.

The reaction was initiated by adding 10 µl of the freshly prepared substrate to either 10 µl of the crude membrane preparation, usually at a concentration equivalent to $0{\cdot}2 \times 10^6$ cells, protein concentration 2 mg/ml, or 50 µl of the cytosol preparation, protein concentration 2 mg/ml. After incubation for 10 min at 37°C the reaction was stopped by the addition of 500 µl 1 N HCl and 2 ml chloroform/methanol (2:1). The mixture was agitated vigorously, the phases separated by brief centrifugation at 250 *g* for 2 min and 500 µl of the upper (aqueous) phase removed for scintillation counting in a LKB1212 Rackbeta liquid scintillation counter. All samples were assayed in duplicate and appropriate reaction blanks were included with each batch. After correction for the blank, activity was calculated as pmol PIP_2 hydrolysed/min/10^6 cells. Protein concentration was determined by the Lowry method.[19]

Nucleotide stimulation

To examine the effect of nucleotide stimulation on PLC activity, the crude membrane preparations were incubated for 10 min with ATP made up to a final concentration of 2 mM and the GTP analogues, GTPγS and GDPβS (Sigma Chemical Co. Ltd, Dorset, U.K.), made up to a final concentration of 2 µM. Substrate concentration was 0·5 nM and PLC activity was measured as before.

Results

Preliminary experiments were performed to determine the optimum pH for PLC activity, using the substrate buffer 20 mM Tris, 1 mM $CaCl_2$, 100 mM KCl, 25 mM LiCl, 1 mM $MgCl_2$, made up at various pHs (Fig. 2). PIP_2 was hydrolysed by membrane PLC over a broad pH range from pH 6 to 8 with optimal activity at pH 6·8. Optimal cetrimide concentration was found to be 0·4 mg/ml.

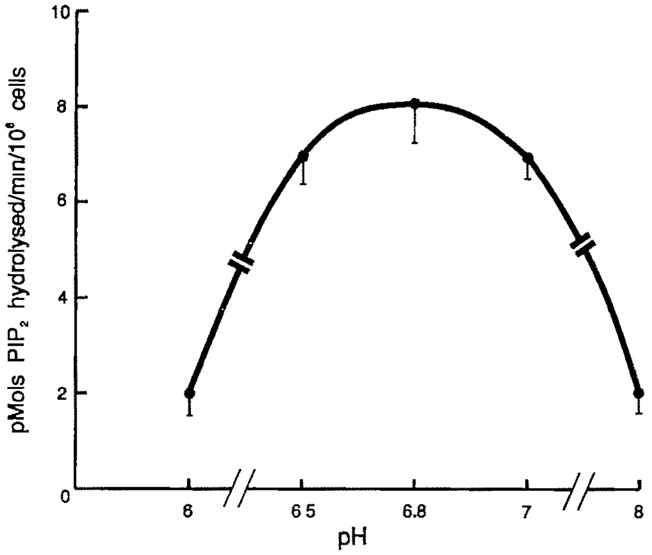

Figure 2. PLC activity using a substrate concentration of 0·5 nM PIP_2 at pH: 6, 6·5, 6·8, 7 and 8. Optimum PLC activity occurs at pH 6·8. Each data point was performed in triplicate, results are mean±SD.

Unstimulated PLC activity in membrane and cytosol preparations

PLC activity was found in both the membrane and cytosol fractions of the MNL preparations from both

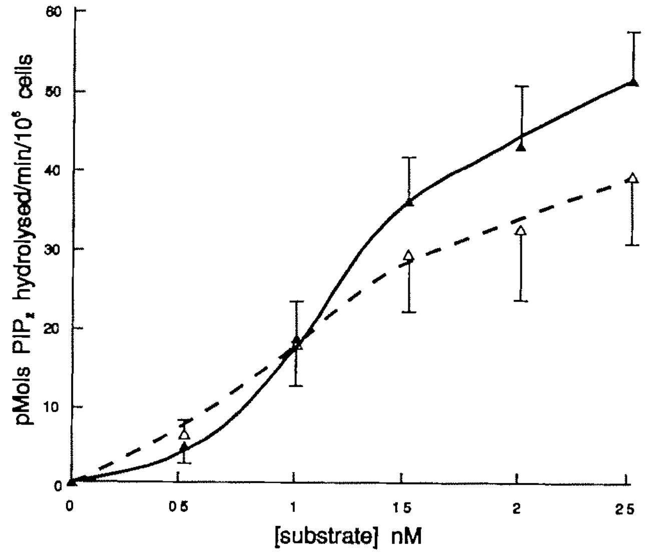

Figure 3. PLC activity in membrane preparations of MNL from subjects with atopic dermatitis and normal controls at increasing concentrations of PIP_2. Results are mean±SD, n=10–13. ▲, atopic; △, normal.

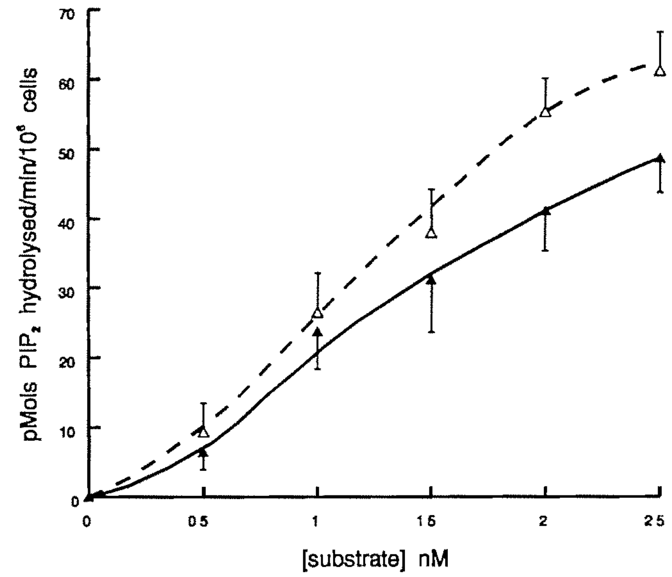

Figure 4. PLC activity in cytosol preparations of MNL from subjects with atopic dermatitis and normal controls at increasing concentrations of PIP$_2$. Results are mean ± SD, n = 7–10. ▲, atopic; △, normal.

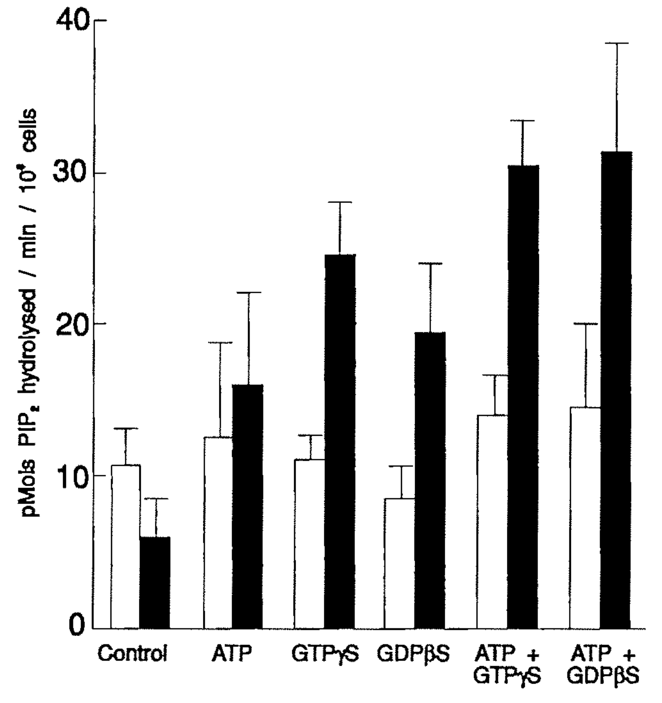

Figure 5. Influence of nucleotides on PLC activity in membrane preparations from subjects with atopic dermatitis and normal controls using a substrate concentration of 0·5 nm. Results are mean ± SD, $n = 5$–7. ☐, normal; ■, atopic.

normal and AD subjects. The response of PLC activity in the membrane preparations of the two groups to increasing substrate concentrations is shown in Figure 3. In both the normal controls and AD subjects a sinusoidal response curve is seen. The response is more exaggerated in AD and at a substrate concentration of 2·0 nmol, PLC activity is significantly higher in AD compared with normals (AD 42·8 ± 7·56 pmol/min/10^6 cells ($n = 14$) Normal 32·3 ± 8·82 pmol/min/10^6 cells ($n = 13$) $P < 0·0025$).

In the cytosol preparations both groups show a more linear increase in PLC activity with increasing substrate concentration, but activity is consistently lower in AD compared with normals (Fig. 4).

Nucleotide stimulation

The effect of nucleotide stimulation on PLC activity in membrane fractions from AD and normals at a substrate concentration of 0·5 nm is shown in Figure 5. A substrate concentration of 0·5 nm was used to reflect the tiny quantities of endogenous substrate normally available. Activity of unstimulated controls is less in AD compared with normals, consistent with the previous results of the unstimulated dose–response curve to substrate concentration. In AD, compared with the unstimulated control, there is a significant increase in PLC activity with ATP, GTPγS, and GDPβS. Maximal increase was seen with the combinations of ATP with both GTPγS and GDPβS. We could only demonstrate significant stimulation of PLC by nucleotides in AD subjects.

Discussion

We have demonstrated PLC activity in MNL from AD and normal subjects using micelle suspensions. This method has not been reported in human leucocytes previously. The finding of an optimum pH of 6·8 is in accordance with the results of studies by Bennet and Crooke on guinea-pig uterus and those by Rebecchi and Rosen on bovine brain.[20,21] The subsequent decrease in PLC activity as the pH of the assay mixture is raised to pH 8 is in accordance with the findings of Bergers *et al.* in rat brain. To optimize the measurement of PLC in human MNL our method differed from that of Bergers in the following ways: sonication of substrate 'cocktail' at 22–30°C instead of 0°C, a change in buffer content for the assay to 20 mm Tris, 1 mm CaCl$_2$, 100 mm KCl, 25 mm LiCl, 1 mm MgCl$_2$, at an optimum pH of 6·8, and a change in chloroform/methanol ratio for separation of lipid and aqueous phase from 1:1 to 2:1. $MgCl_2$ was added to the assay buffer solution because the degree of phosphorylation of PIP_2 has a profound effect on the enzyme's requirement for Mg^{2+} ions.[22] LiCl was added to inhibit dephosphorylation of inositol phosphate to inositol.[23]

The reduced activity of AD–PLC in both membrane and cytosol fractions at 0·5 nM substrate concentration compared with normals may partly be a consequence of a greater pool of intracellular PIP_2, but previous studies have shown that resting inositol turnover in atopics is little different from normals.[16]

Hydrolysis of PIP_2 occurred over a wide range of substrate concentrations in membrane and cytosol fractions of MNL from both AD and normal subjects. The sinusoidal response curves seen in the membrane fractions are consistent with either a mixture of isoenzymes or an enzyme with multiple active binding sites.

The decrease in cytosolic PLC activity in AD compared with normals may reflect migration of the enzyme between cellular compartments, or could suggest several PLC isoenzymes within the cell.

The stimulation of membrane-bound PLC in AD by all the nucleotides suggests that this was a non-specific effect rather than mediated via a G protein, in which event stimulation would have been confined to the non-hydrolysable GTP analogue GTPγS. Sommermeyer *et al.* demonstrated similar results in rabbit thymocytes when using exogenous substrate, but also demonstrated specific G protein-mediated stimulation when only endogenous substrate was available.[24] Thus the response of membrane-bound PLC to G protein stimulation appears to be assay dependent. The non-specific stimulation seen in the presence of exogenous substrate may imply that the response is due to a local increase in substrate concentration or may be a direct effect on PLC resulting in conformational change of the enzyme, thereby increasing activity.

The reason for the marked difference in response to both increased substrate concentration and to nucleotide stimulation of membrane PLC between normals and AD is at present unknown. It may reflect activation of the PLC system in the MNL in AD as a consequence of abnormal cytokine production *in vivo*. Alternatively it may suggest that membrane-bound PLC in AD is a more active isoenzyme of normal PLC.

Our study suggests that further investigation of PLC using different assay methods including immunoquantification will be required to confirm a functional abnormality of this system in AD.

Acknowledgments

Parts of this paper were presented at the British Society of Investigative Dermatology meeting in Newcastle, U.K. in September 1990, and at the European Society of Dermatological Research meeting in Copenhagen, Denmark in June 1991.

We are grateful to the National Eczema Society for financial support for Miss S.Myint.

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British Journal of Dermatology (1992) **127**, 103–111.

The distribution of $\alpha6\beta4$ integrins in lesional and non-lesional skin in bullous pemphigoid

V.A.VENNING, J.ALLEN,* J.D.APLIN, G.KIRTSCHIG AND F.WOJNAROWSKA

Department of Dermatology, Slade Hospital, Oxford

**Department of Obstetrics and Gynaecology, University of Manchester, U.K.*

Accepted for publication 14 April 1992

Summary

The $\alpha6\beta4$ integrin is associated ultrastructurally with the hemidesmosomes of the basal keratinocytes and with the bullous pemphigoid antigen (BPA), suggesting an important role in adhesion of epidermal cells to the basement membrane. Using an immunofluorescence technique with chain-specific monoclonal antibodies to the α and β subunits we have investigated the distribution of the $\alpha6\beta4$ integrin in normal skin ($n=3$) and in BP skin (uninvolved, perilesional and lesional) [$n=11$]). The findings have been compared with other types of subepidermal blisters and with normal skin split by chemical means ($n=2$) and by suction ($n=2$). The distribution of $\alpha6\beta4$ integrin was compared with that of bullous pemphigoid antigen (BPA) and with other basement membrane zone (BMZ) macromolecules, laminin, collagen type IV, collagen type VII and the BM600 antigen. In uninvolved, perilesional and early pre-blistered lesional BP skin the distribution of both the $\alpha6$ and $\beta4$ integrin subunits, BPA laminin, collagen types IV and VII and the BM600 antigen was identical to normal skin, i.e. a linear band in the BMZ. Within BP blisters, both $\alpha6$ and $\beta4$ integrin subunits and BPA were absent, except in two blisters in which the integrin expression was retained in the blister roof, despite loss of BPA. The other BMZ components were expressed on the blister floor. These findings distinguished BP from linear IgA disease (patchy retention of $\alpha6\beta4$ integrin expression along the blister roof and floor, with BPA expressed in the roof), and from blisters of the dermolytic type (recessive dystrophic epidermolysis bullosa and eosinophilic cellulitis) in which all the BMZ components, including $\alpha6\beta4$ integrin and BPA were expressed along the blister roof. In artifically split skin, $\alpha6\beta4$ integrin and BPA were expressed along the roof of the split, whereas the other BMZ components were expressed along the floor. We conclude that although there is no widespread abnormality of $\alpha6\beta4$ integrin expression in uninvolved BP skin, nor in early pre-blistered lesions, loss of expression of both $\alpha6\beta4$ integrin and BPA is a feature of most fully developed BP blisters, and is specific for BP compared with other types of subepidermal blisters. Disruption of the hemidesmosome-related antigens appears be a prerequisite for blistering to occur in BP.

Bullous pemphigoid (BP) is a subepidermal blistering disorder, the blisters resulting from separation of the basal epidermal cells from their underlying basement membrane. In BP a circulating antibody binds to the basement membrane zone at the dermo-epidermal junction, and blisters result from separation at this site.[1] The major target antigen of the BP antibody is a 220-kDa polypeptide,[2-4] less commonly a 180-kDa polypeptide,[4-12] and the BP target antigens are closely associated with epidermal hemidesmosomes,[13-18] structures crucial for adhesion of the epidermis to the underlying dermis.[19]

Integrins are a family of heterodimers comprising two non-covalently associated transmembrane glycoproteins, α and β. Some integrins bind to other cell-surface proteins thereby mediating cell–cell interactions, whereas others have extracellular matrix ligands and mediate cell–matrix interactions.[20-22] Extracellular matrix ligands for integrins include laminin, fibronectin and various collagens.[23] At least three integrins are associated with normal epidermis, $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha6\beta4$, all predominantly in the basal layer.[24] Integrins $\alpha2\beta1$ and $\alpha3\beta1$ are located at the lateral and basal aspects of the basal cells, whereas $\alpha6\beta4$ is confined to the basal pole, where the basal keratinocytes abut the basement membrane zone (BMZ). Recently, ultrastructural studies have shown that in skin[25] and cornea[26] the $\alpha6\beta4$ integrin is located in hemidesmosomes, closely associated with the

Correspondence: Dr V.A.Venning, Department of Dermatology, Slade Hospital, Oxford, U.K.

BP antigen (BPA). This distribution suggests a major role for the $\alpha6\beta4$ integrin in keratinocyte–basement membrane zone adhesion, and interference with this function is likely to be implicated in the pathogenesis of subepidermal blistering.

Using chain-specific monoclonal antibodies to the α and β subunits and an immunofluorescent technique, we have investigated the distribution of $\alpha6\beta4$ integrin in lesional and non-lesional BP skin and compared the findings with other types of subepidermal blisters and with normal skin.

Methods

Patients

Specimens were examined from 11 untreated patients with histologically and immunologically proven bullous pemphigoid, one with linear IgA disease (LAD), one with recessive dystrophic epidermolysis bullosa (RDEB), and one with eosinophilic cellulitis with blisters. Punch biopsies (4 mm) were taken from lesional and uninvolved skin under local anaesthetic, snap frozen in liquid nitrogen and stored at −70°C until needed. In three of the 11 BP patients the lesion studied was a plaque of urticated erythema. In the eight remaining patients blisters were studied.

Controls

Frozen normal skin samples obtained from surgical specimens ($n=3$) and skin artificially split by suction ($n=2$) or by incubation with 1 M NaCl ($n=2$) were used as controls. Skin split by these means separates in the same ultrastructural plane as BP blisters, i.e. through the lamina lucida of the basement membrane.[27] Suction blisters were raised on the volar aspect of the volunteer's forearm using a glass suction cup with holes of 5 mm diameter. The cup was attached to the forearm and connected to an Eschmann CP50 suction pump operating at a negative pressure of 200 mmHg. The resulting blister was removed, when still small, by 4-mm punch biopsy under local anaesthetic, snap frozen in liquid nitrogen and stored at −70°C until needed. Saline-split skin was obtained from fresh normal skin (surgical specimens), incubated for 24–48 h in 1 M NaCl at room temperature and the epidermis gently lifted from the dermis with forceps.

Immunohistochemistry

The skin specimens were incubated with a panel of monoclonal antibodies (MAbs) specific for integrin subunits and other basement membrane zone components (Table 1). The MAb GoH3 is specific for the $\alpha6$ integrin chain,[28] and G71[29] and 5B5[25] are specific for the extracellular domain of the $\beta4$ integrin chain. The bullous pemphigoid antigen was detected by using a BP serum shown by immunoblotting to be specific for the 220-kDa BP antigen. Commercially available antibodies (DAKO) specific for laminin and type IV collagen were used. The MAb LH 7·2 recognizes an epitope of the type VII collagen molecule and localizes to the anchoring fibrils of the upper dermis[30] (Gift of Dr Irene Leigh). MAb GB3 (Sera-lab) was originally raised against the basement membrane of human amnion but also recognizes BM 600, a 600-kDa glycoprotein component of skin BMZ.[31,32]

Cryosections (5 μm) of skin were washed in PBS and air-dried prior to incubation for 1 h at room temperature with the relevant monoclonal antibody. The primary antibody was removed with 3 × 15-min washes in PBS and the sections were then incubated with fluorescein-labelled rabbit anti-mouse, or swine anti-rabbit antibody, (as appropriate) for 1 h at room temperature. After further washing the sections were mounted in Citifluor and specific binding was demonstrated by fluorescence microscopy. Each run included a negative control by omission of the first step antibody.

Table 1. Panel of antibodies used and their specificities

Antibody	Specificity
G71	$\beta4$ integrin chain
5B5	$\beta4$ integrin chain
GoH3	$\alpha6$ integrin chain
BP serum	220-kDa BP antigen
Anti-laminin (DAKO)	Laminin
Anti-collagen type IV (DAKO)	Collagen type IV
LH 7 2	Anchoring fibrils/collagen type VII
GB3 (Sera-Lab)	BM600

Results

The results are summarized in Table 2.

Intact normal skin

Each of the MAbs gave an identical band of linear fluorescence in the BMZ in intact normal skin.

Table 2. Reactivities of skin sections to the panel of antibodies

Diease (no. of specimens)	Antibody against						
	$\alpha6$ integrin subunit	$\beta4$ integrin subunit	BPA (220 kDa)	Laminin	Type IV collagen	Type VII collagen	BM 600 antigen
BP							
Uninvolved (11)	Linear BMZ	Linear BMZ	Linear BMZ (10)	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ
Urticated erythema (3)	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ
Blister (8)	Absent (7) + roof (1)	absent (6) + roof (2)	Absent (8)	+ floor (8)	+ floor (8)	+ floor (8)	+ floor (7) + roof and floor (1)
LAD							
Uninvolved (1)	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ
Blister (1)	Flecks + in roof and floor	Flecks + in roof and floor	+ roof	+ floor	+ floor	+ floor	+ floor
RDEB							
Uninvolved (1)	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ
Blister (1)	+ roof	+ roof	+ roof	+ roof	+ roof	patchy + roof and floor	+ roof
Eosinophilic cellulitis							
Uninvolved (1)	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ
Blister (1)	+ roof	+ roof	+ roof	+ roof	+ roof and floor	+ roof	+ roof
Controls							
Normal skin (3)	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ	Linear BMZ
NaCl split skin (2)	+ roof	+ roof	+ roof	+ floor	+ floor	+ floor	+ floor
Suction blister (2)	+ roof	+ roof	+ roof	+ floor	+ floor	+ floor	+ floor

BP, bullous pemphigoid; LAD, linear IgA disease; RDEB, recessive dystrophic epidermolysis bullosa.

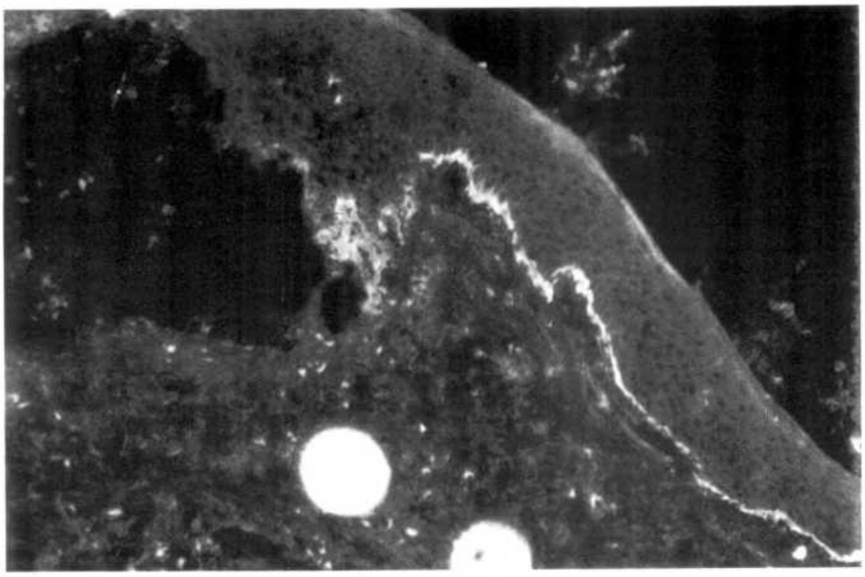

Figure 1. Bullous pemphigoid blister incubated with anti-$\beta 4$ antibody (G71) and fluorescein-labelled anti-mouse second antibody. Linear fluorescence along the BMZ in perilesional skin is abruptly lost at the blister edge (original magnification × 100).

Bullous pemphigoid

Uninvolved BP skin was identical to normal control skin with each of the MAbs. In lesions comprising urticated erythema without a blister, the results were also identical with normal control skin. In BP blisters the anti-integrin MAbs GoH3, G71 and 5B5 had similar distributions in each case. In six blisters linear BMZ fluorescence with each of the anti-integrin MAbs was present in the perilesional skin, but was abruptly lost at the blister edge (Fig. 1). In one blister fluorescence with three of the anti-integrin MAbs was retained along the length of the blister roof, and in one blister there was patchy expression along part of the roof with the anti-$\beta 4$ MAbs G71 and 5B5. The anti-220-kDa BP serum gave positive fluorescence up to the blister edge in all but one case, but was absent within the blister in all eight cases. Antibodies to laminin (Fig. 2) and collagen types IV and VII (Fig. 3) produced fluorescence along the blister floor. MAb GB3 also produced floor fluorescence in 7/8 blisters (Fig. 4), but in one blister GB3 gave both roof and floor fluorescence.

Non-BP blisters

In all the uninvolved skin specimens the MAbs each gave a linear band of BMZ fluorescence.

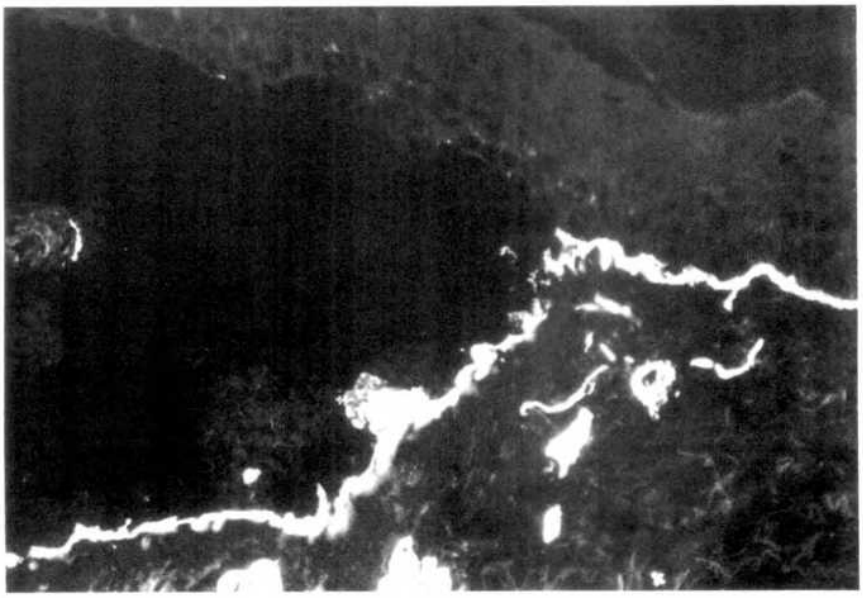

Figure 2. Bullous pemphigoid blister incubated with anti-laminin antibody and fluorescein-labelled anti-rabbit second antibody. Linear fluorescence along the BMZ in perilesional skin is retained along the blister floor (original magnification × 100).

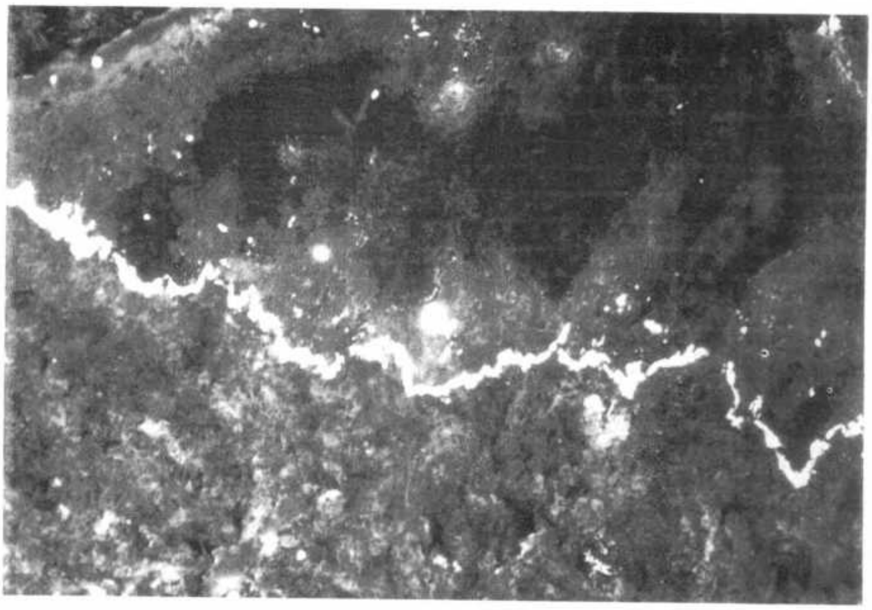

Figure 3. Bullous pemphigoid blister incubated with antibody specific for type VII collagen (LH 7·2) and fluorescein-labelled anti-mouse second antibody. Linear fluorescence along the BMZ in perilesional skin is retained along the blister floor (original magnification × 100).

Within the blister of the patient with linear IgA disease the anti-integrin MAbs GoH3, G71 and 5B5 had identical distributions, with flecks of persistent fluorescence in both roof and floor. The 220-kDa-BP serum gave continuous positive fluorescence up to the blister edge, which persisted within the lesion, lying along the blister roof. Antibodies to laminin and collagen types IV and VII and the MAb GB3 all produced fluorescence along the blister floor.

In recessive dystrophic epidermolysis bullosa, anti-integrin MAbs GoH3, G71 and 5B5 produced fluorescence along the blister roof. The 220-kDa BP serum and the antibodies to laminin and collagen types IV and VII and the MAb GB3 also produced fluorescence along the blister roof, although flecks of fluorescence were also seen on the blister floor with type VII collagen.

In eosinophilic cellulitis the anti-integrin MAbs GoH3, G71 and 5B5 produced fluorescence along the blister

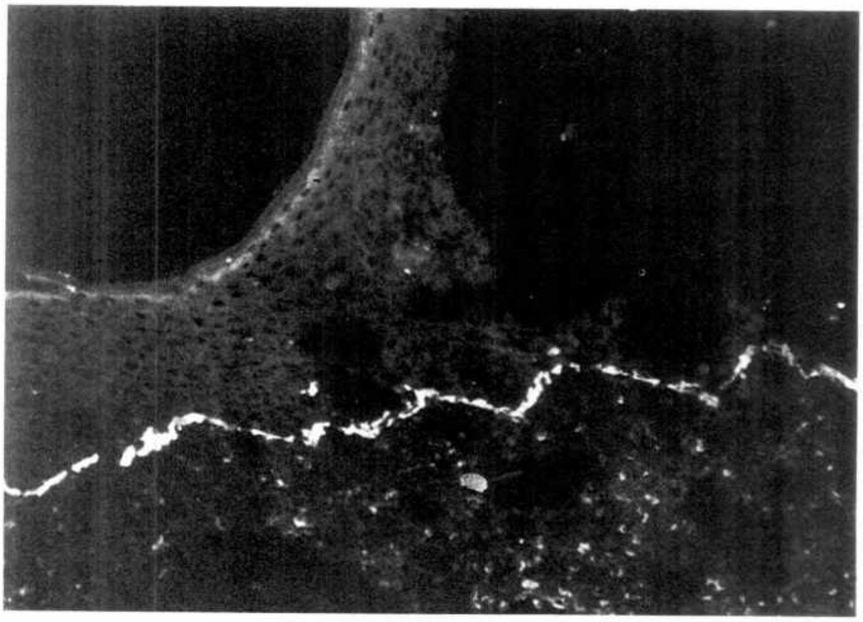

Figure 4. Bullous pemphigoid blister incubated with antibody specific for the BM 600 antigen (GB3) and fluorescein-labelled anti-mouse second antibody. Linear fluorescence along the BMZ in perilesional skin is retained along the blister floor (original magnification × 100).

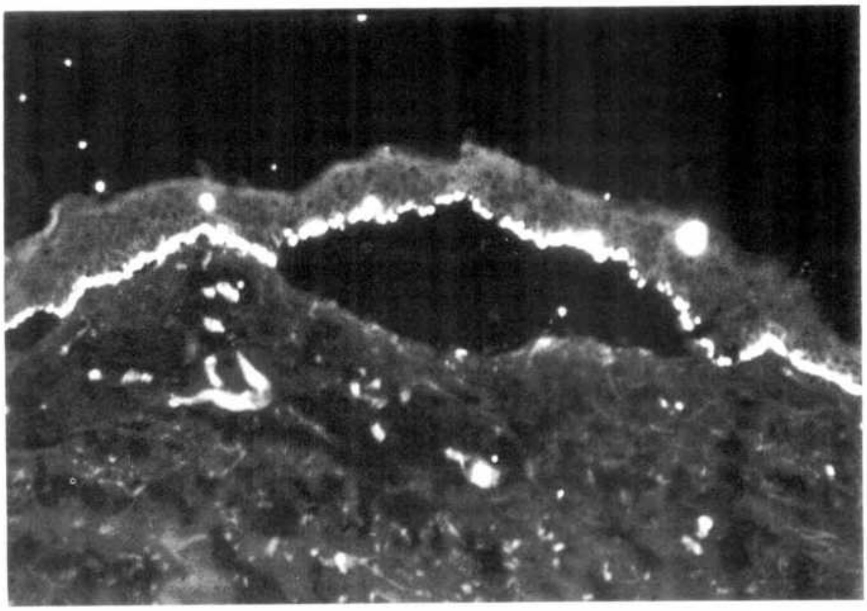

Figure 5. Suction blister from a normal volunteer incubated with anti-$\beta4$ antibody (G71) and fluorescein-labelled anti-mouse second antibody. In contrast with BP, expression of the integrin chain is retained in the blister roof (original magnification × 100).

roof. The 220-kDa BP serum and the antibodies to laminin and collagen types IV and VII and the MAb GB3 also produced fluorescence along the blister roof, although flecks of fluorescence were seen on the blister floor with type IV collagen.

Artificially split normal skin

Split-skin specimens, whether separated by suction or by chemical means, gave identical results. The anti-integrin MAbs GoH3, G71 (Fig. 5) and 5B5 and the anti-BP serum produced fluorescence along the epidermal aspect of the split, whereas the antibodies to laminin and collagen types IV and VII and the MAb GB3 produced fluorescence along the floor of the split.

Discussion

The $\alpha6\beta4$ integrin occurs in most epithelial cells including epidermis.[28] It is localized ultrastructurally to the hemidesmosomes along the basal pole of the basal keratinocytes,[25,26] suggesting an important function mediating adhesion of the epidermis to the underlying BMZ. Both the $\alpha6$ and $\beta4$ subunits are transmembrane glycoproteins, presumably linking an intracytoplasmic ligand to one within the basement membrane, although these have yet to be identified. $\beta4$ has an unusually long intracytoplasmic domain which must contribute to the structure of the hemidesmosomal dense plaque on the cytoplasmic side of the cell membrane.[25,33,34] Our results with the anti-$\alpha6$ and two anti-$\beta4$ antibodies were similar. Keratinocytes express these subunits as the heterodimer $\alpha6\beta4$,[25] and there is no evidence for association of either $\alpha6$ or $\beta4$ with other integrin subunits in the epidermis. It is therefore likely that the distribution of each subunit reflects that of the heterodimer. The bullous pemphigoid antigen (BPA) localizes intracellularly, also in association with the hemidesmosome,[13] and it has been suggested that rather than occupying a position within the dense plaque immediately adjacent to the cell membrane, the BPA is situated between the dense plaque and the inner plaque where the intermediate filaments insert.[13,25,35]

Although the exact relationships between the various structural components of the hemidesmosome and their intra- and extracellular ligands is unknown, this molecular complex presumably normally contributes to stable dermo-epidermal adhesion, and in cell culture models anti-$\alpha6\beta4$ monoclonal antibodies promote detachment of cells from their substratum.[36] The development of a blister in the upper BMZ such as occurs in BP, suggests possible loss of continuity of the molecular structures normally responsible for dermo-epidermal adhesion, either by disruption of the integrin molecule or by its dissociation from intra- or extracellular ligand. Our results confirm the recent report of Michalaki *et al.*[37] that $\alpha6\beta4$ is absent within BP blisters, and further shows that this is associated with loss of the BPA, suggesting disruption of the BPA-$\alpha6\beta4$ complex within the hemidesmosome.

In uninvolved skin from patients with BP the distribution of the $\alpha6$ and $\beta4$ integrin chains was identical with normal skin, as was that of other macromolecular components of the BMZ. This excludes any widespread defect of $\alpha6\beta4$ expression in the cutaneous BMZ acting as an underlying factor predisposing to blistering. However, within all but one of the BP blisters both the $\alpha6$ and $\beta4$ integrin chains were absent, and BPA was similarly absent from these blisters. Monoclonal antibodies to different epitopes would be necessary to distinguish whether the loss of integrin and BPA expression in BP blisters is due to physical loss of the molecules in blistered skin or to conformational changes or proteolytic modification preventing recognition of the epitopes by the antibodies used in this study. Ultrastructurally, hemidesmosomes are absent in fully developed BP blisters,[38] although it is unknown whether the constituent molecules persist despite no longer being organized into electron-dense structures. Conformational changes in $\alpha6\beta4$ secondary to antibody binding to the closely adjacent BPA, is insufficient to account for the loss of integrin expression which, despite widespread antibody binding to BPA, was not seen in non-blistered skin. The integrin chains and BPA were normally expressed in the perilesional BMZ as far as the blister edge, where fluorescence to the relevant antibody abruptly disappeared. Furthermore, within BP lesions consisting only of plaques of urticated erythema, such as commonly precede the development of overlying blisters, there was also normal expression of the $\alpha6$ and $\beta4$ integrin chains and BPA, suggesting that 'loss' of integrins and BPA in BP is a feature only of skin areas where there is total loss of adhesion between the basal cell and the underlying BMZ.

Loss of integrin chain fluorescence occurred in all BP blisters with only one exception, in whom both chains were consistently retained along the blister roof in repeated experiments. The BPA, however was not detected within the same blister, suggesting that, despite their close physical proximity BPA and $\alpha6\beta4$ may be independently affected. There were no apparent clinical or other differences between this one patient and the others, and the specificity of this patient's target antigen could not be determined, as Western blotting of the serum against an epidermal extract produced negative results.

Type VII collagen, a component of the sublamina densa anchoring fibrils,[30] localized to the floor of BP blisters, as did laminin and type IV collagen, which are both components of the lamina densa.[39,40] These findings are commensurate with the ultrastructural site of BP blisters through the lamina lucida.[38]

The loss of expression of the hemidesmosome-related antigens in BP blisters is not a non-specific finding and does not occur in all types of subepidermal blisters. LAD, characterized by an IgA class anti-BMZ antibody, has a high molecular weight target antigen of 285 kDa.[41] The blister in this study was distinguished from BP blisters in having retention of the integrin chains and BPA in flecks along both the roof and floor of the blister, and also of BPA in flecks along blister the roof. The lower BMZ macromolecules, laminin and collagen types IV and VII formed the floor, a finding which suggests a split high in the BMZ but not resulting in total loss of the hemidesmosome-related antigens. LAD blisters have been ultrastructurally localized by different authors to the lamina lucida, lamina densa and sublamina densa regions,[41] suggesting the disease may be heterogeneous.

Blisters which develop in the lower BMZ, physically distant from the hemidesmosomes are associated with no alteration in integrin expression. The pattern of fluorescence in the RDEB case was consistent with the site of blistering through the upper dermis, with the integrin chains as well as the components of the lower BMZ forming the blister roof. Similarly, in eosinophilic cellulitis, in which the blisters form by lifting of the epidermis in areas of tense upper dermal oedema, all the BMZ components appeared, as predicted, in the blister roof.

In artifically separated skin, whether split by chemical means or by suction, the plane of cleavage is through the lamina lucida. Despite apparent proximity to the site of the split in BP blisters, integrin and BPA expression was unaltered in the artificial blisters, and both components of the hemidesmosomes were expressed in the roof while the lower BMZ components resided in the floor. These findings differ slightly from those of Michalaki *et al.*[37] who observed that although $\alpha6\beta4$ integrin was present in salt-split skin, the staining was altered and patchy. The reasons for this discrepancy are unclear.

The intra- and extracellular ligands of $\alpha6\beta4$ integrin are as yet unknown. It has been suggested that intracellularly $\alpha6\beta4$ may be associated with the intermediate filaments of the cytoskeleton, either directly or indirectly through the BPA.[25] An association between keratin filaments and the BPA has been proposed[42] and it has been further suggested that the BPA may be broadly analogous to desmoplakins, with which it shares some structural homology.[43] Desmoplakins occupy a comparable position within the desmosome[44] to that described for BPA within the hemidesmosome, i.e. between the membrane associated plaque and the intermediate filaments.[13,25,35]

The extracellular ligand of $\alpha6\beta4$ is presumably a molecular component of the lamina lucida of the BMZ. On colon cancer cell lines $\alpha6\beta4$ may act as a laminin receptor[45] although no evidence for this function was found for $\alpha6\beta4$ on mammary cell lines[28] and it is possible that the ligand varies in different tissues. In this study MAb GB3 was used to detect a BMZ glycoprotein, BM600, residing in the region of the anchoring filaments.[31,32] BM600 has been only partially characterized, but may correspond to kalinin[46] or to epiligrin,[47] recently described components of anchoring filaments and potential candidates for the extracellular ligand of $\alpha6\beta4$ integrin. In every type of blister studied the BM600 antigen was expressed, usually along the blister floor, except in the case of dermolytic blisters where it was expressed along the roof. Interestingly, in the single BP patient in whom integrin expression was retained along the blister roof, GB3 was co-distributed in areas of the blister roof in addition to the principle site of GB3 positivity in the blister floor. The partial co-localization of BM600 with $\alpha6\beta4$ integrin in this BP patient suggests it may be a potential candidate for the extracellular ligand of $\alpha6\beta4$. However, conformational changes consequent upon dissolution of any ligands between $\alpha6\beta4$ and BM600 did not result in loss of GB3$^+$ epitopes, as positivity was retained, even under conditions when the hemidesmosome-related antigens were no longer expressed.

We have shown that in uninvolved skin in BP there is normal expression of each of the hemidesmosome components, BPA and the integrin chains, $\alpha6$ and $\beta4$. However, blisters in BP usually form with accompanying loss of antibody recognition of all three components of the hemidesmosome, a finding which was specific to BP among the subepidermal blisters studied. Loss of expression of the hemidesmosome-related antigens is not a feature of perilesional skin and early pre-blistered BP lesions, suggesting that although not a primary event in the pathogenesis of BP inflammatory lesions, hemidesmosome disruption is a prerequisite for the formation of BP blisters.

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Ultrastructural immunogold studies in two cases of linear IgA dermatosis. Are there two distinct types of this disease?

S.KÁRPÁTI,*† W.STOLZ,† M.MEURER,† T.KRIEG†‡ AND O.BRAUN-FALCO†

** Dermatology Department, Heim Pál Hospital for Children, Budapest, Hungary*
† Dermatology Department, Ludwig-Maximilians University Munich, Germany
‡ Dermatology Department, University Köln, Germany

Accepted for publication 15 January 1992

Summary

It has been suggested that patients with homogeneous linear IgA deposits at the basement membrane zone constitute a distinct bullous disorder called linear IgA dermatosis (LAD) of adults or children. The results of the present ultrastructural immunogold study in two patients with LAD suggest that LAD is not a single disease entity. LAD in a 10-year-old girl was found to be ultrastructurally similar to an IgA-type pemphigoid. IgA was detected in the uppermost lamina lucida underlying the basal cell plasma membrane. In a second patient, an 86-year-old man, IgA deposits were present within the lamina densa and the anchoring plaques. The distribution of IgA in this patient was ultrastructurally identical with that of IgG in epidermolysis bullosa acquisita skin and with that of the non-collagenous globular terminus of collagen VII within the basement membrane zone of normal skin. By using the immunogold technique, we could distinguish two distinct types of LAD according to the IgA binding sites in the diseased skin. We suggest that different labelling patterns may correspond to different clinical pictures.

Chorzelski, Jablonska and Beutner suggested that patients with homogeneous linear IgA deposits at the dermo-epidermal junction constituted a distinct disease entity to be differentiated from dermatitis herpetiformis and bullous pemphigoid.[1,2] The disease was called chronic bullous disease of childhood or linear IgA dermatosis (LAD) of childhood and adults.

Some patients with LAD have a blistering skin disease that heals without scars and has a good prognosis,[3,4] others show severe cicatricial mucosal involvement, especially ocular disease, and eventually develop conjunctival scarring.[4–6] Adult patients may occasionally develop lymphomas, and, rarely, other malignancies.[7,8]

Immunoelectron microscopic (IEM) studies using the peroxidase technique have demonstrated that there are LAD patients who have IgA deposited in the lamina lucida,[9–15] adjacent to the plasma membrane of basal keratinocytes[9–13] or in the lower part of the lamina lucida.[14] In other cases, IgA was found in the sublamina densa region.[9–11,15–18] There are also reports of patients with LAD, however, who had IgA both within the lamina lucida and within the sublamina densa region.[10,12,15] Because the peroxidase technique permits only a rough pattern analysis, a detailed visualization of the structures involved in the several types of LAD was not possible. By using the more advanced immunogold technique in LAD, we could clearly differentiate ultrastructurally two distinct antibody binding sites in the skin of two patients.

Methods

Patients

Patient 1 was a 10-year-old girl with a 1-month history of blisters appearing on the face and trunk, which showed the typical 'cluster of jewels' distribution. There was no mucosal involvement, and the blisters healed without scars. A biopsy was taken of a small blister on the trunk. Direct immunofluorescence studies revealed linear IgA and C3 along the basement membrane zone (BMZ). Indirect immunofluorescence was negative.

Patient 2 was an 86-year-old man with a 4-month history of an intensely pruritic rash. At the time of admission to hospital, there were disseminated papular and vesicular lesions, involving the face, trunk and limbs. Later in the disease course he developed large, tense blisters and erosions which covered most of the body, and healed without scarring. There was no

Correspondence: Dr S.Kárpáti, NIH, Dermatology Branch of the National Cancer Institute, Bethesda, MD, 20892 U.S.A.

mucosal involvement. General examination did not reveal any evidence of an underlying malignancy. Histology of a skin biopsy from the lower leg revealed a subepidermal blister with a mixed inflammatory infiltrate of neutrophils and eosinophils within the lumen of the blister and in the adjacent tissue. Direct immunofluorescence showed a thick, linear deposition of IgA along the BMZ. Conventional indirect immunofluorescence microscopy using monkey oesophagus and normal human skin as substrates was negative. A circulating antibody binding to the BMZ on the dermal side of 1M NaCl split skin was detected in a titre of 1:10.

Immunoelectron microscopy (IEM)

Pre-embedding IEM was processed on unfixed tissue samples as reported previously.[19] Briefly, 0·5 × 0·5-mm skin samples from both patients and from normal human skin were incubated overnight at 4°C with mouse anti-human IgA first antibody (Zymed, San Francisco, CA, U.S.A.) diluted in a ratio of 1:15 in phosphate-buffered saline (PBS, pH 7·4, 0·01 M). The specimens were then washed three times for 2 h in PBS and incubated with gold-labelled goat anti-mouse second antibodies (Amersham Buchler, Braunschweig, Germany) diluted in Tris buffer (Tris 20 mmol/l, sodium nitrite 20 mmol/l, pH 8·0) in a ratio of 1:10 and incubated for 5 h at room temperature. Control samples were stained by monoclonal mouse antibodies specific to the globular ends of type VII collagen, diluted 1:10 (a kind gift of Dr Lynn Sakai, Portland, OR, U.S.A.) [Patient 1 and normal human skin] as well as by monoclonal mouse antibodies to anti-human IgM (Zymed), diluted 1:15 [Patient 2]. Tissue samples were post-fixed in Karnovsky solution and in 1% osmium, embedded in epoxy resin (Epon) and processed for ultrastructural studies.

Results

Patient 1

In lesional skin, split formation was found within the lamina lucida. In skin samples exposed to anti-human IgA, immunogold was deposited within the lamina lucida, underlying the basal keratinocyte plasma membrane. There was no labelling along the lamina densa or below it (Fig. 1a and b). The anchoring fibrils were also free of gold particles. In control samples exposed to antibodies against collagen VII, scattered gold particles were detected within the lamina densa and there was heavy labelling within the anchoring plaques (Fig. 2b).

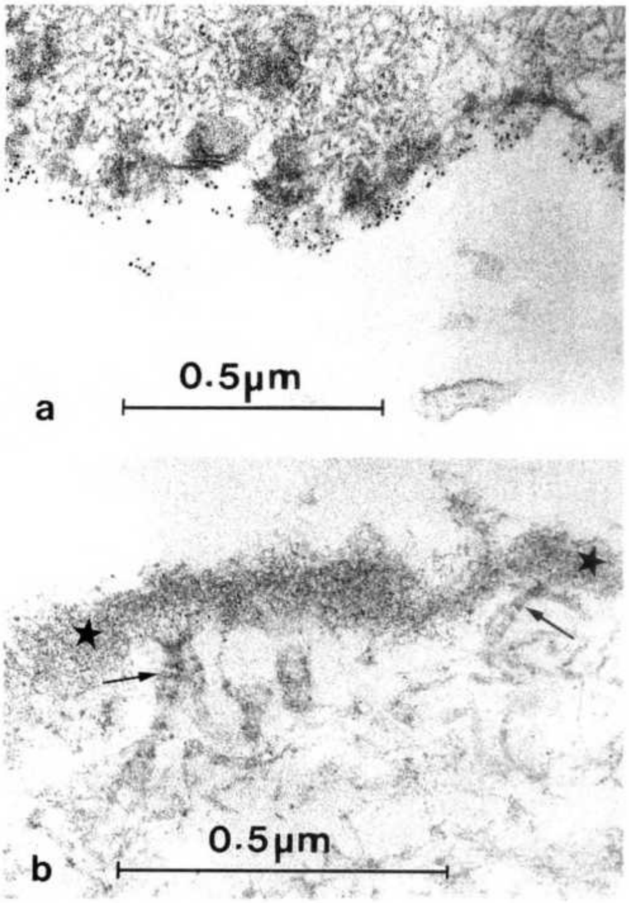

Figure 1. Patient 1. Blister formation; skin samples exposed to anti-human IgA. Note immunogold deposition on the roof (a) but not on the base (b) of the blister. (a) Gold particles underlying the plasma membrane of basal keratinocytes. (b) No labelling along the anchoring fibrils (arrows) [stars indicate the lamina densa] (a × 76,600; b × 94,400).

Patient 2

Perilesional skin. In samples exposed to anti-human IgA there was heavy staining along the dermal ends of anchoring fibrils within the anchoring plaques, as well as scattered gold deposition along the lamina densa (Fig. 3a and b). In some areas the upper surface of the lamina densa (the uppermost ends of anchoring fibrils?) was also labelled (Fig. 2a). The cross-banded anchoring fibrils themselves were free of gold particles. Similar gold distribution was observed in normal human skin stained by antibodies against the globular terminus of collagen VII (Fig. 3c).

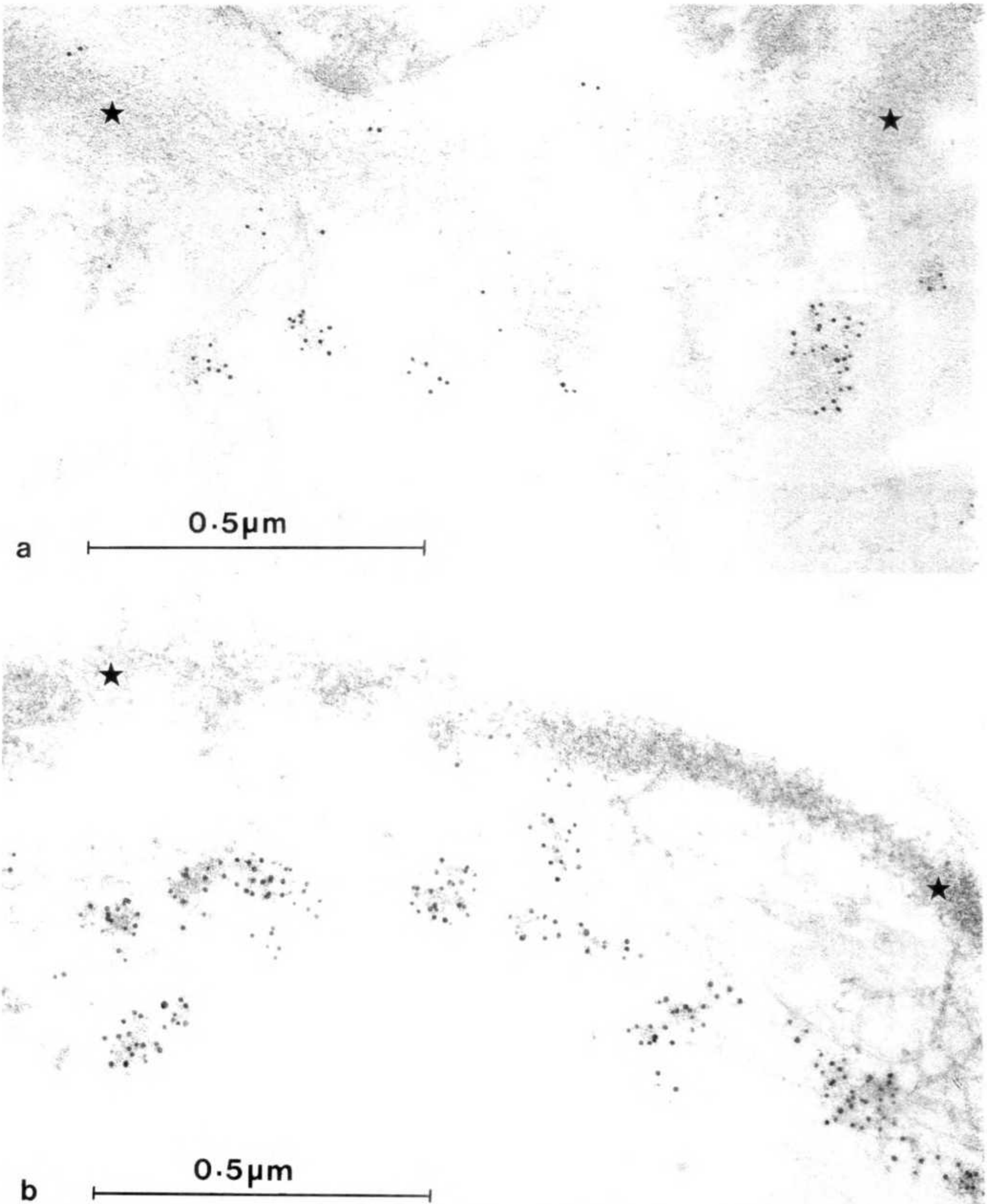

Figure 2. Distribution of gold particles within the basement membrane zone of skin from Patient 2 exposed to anti-human IgA (a), and that from Patient 1 exposed to antibodies against the globular terminus of collagen VII (b). Note the similar ultrastructural labelling. Also note gold particles at the lamina lucida contact surface of the lamina densa, above the anchoring fibrils (a). Stars indicate the lamina densa (a × 160,000; b × 160,000).

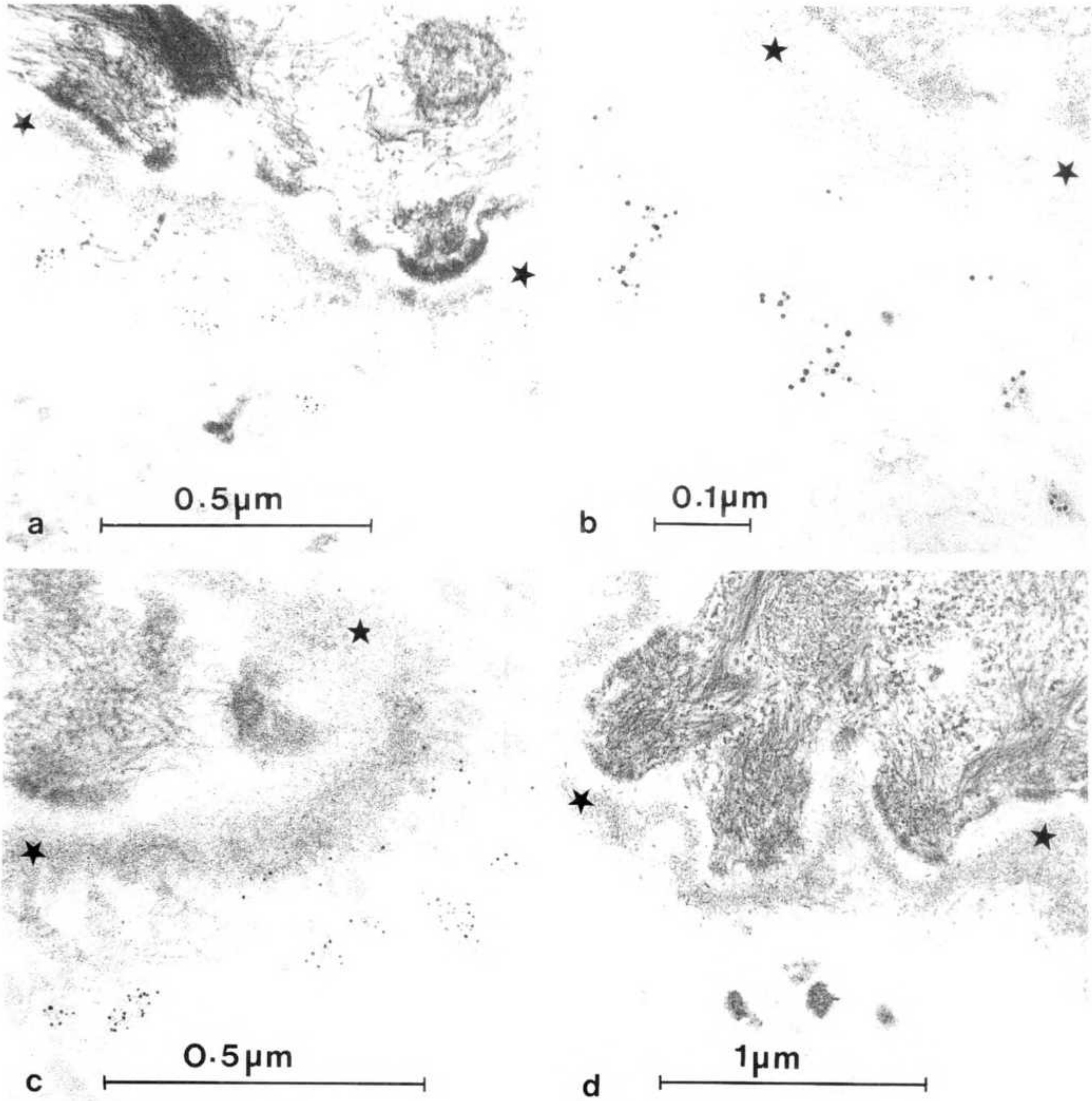

Figure 3 (a and b). Patient 2, perilesional skin, exposed to anti-human IgA. Note gold deposition along the anchoring plaques and also scattered along the lamina densa (stars). (c) Normal human skin exposed to antibodies to the globular terminus of collagen VII. Note similar staining pattern to (a) and (b). (d) Control skin sample from Patient 2 with linear IgA dermatosis. Note blister formation under the lamina densa (stars). (a × 55,500; b × 160,000; c × 109,600; d × 46,000).

Lesional skin. Split formation occurred below the lamina densa, within or underlying the zone of anchoring fibrils (Fig. 3d). Gold deposition was observed within the anchoring plaques and scattered along the lamina densa. In some areas gold particles were also detected on the upper surface of the lamina densa, above the anchoring fibrils (Fig. 4a and b). There was no deposition of gold along the hemidesmosomes. Again, the labelling

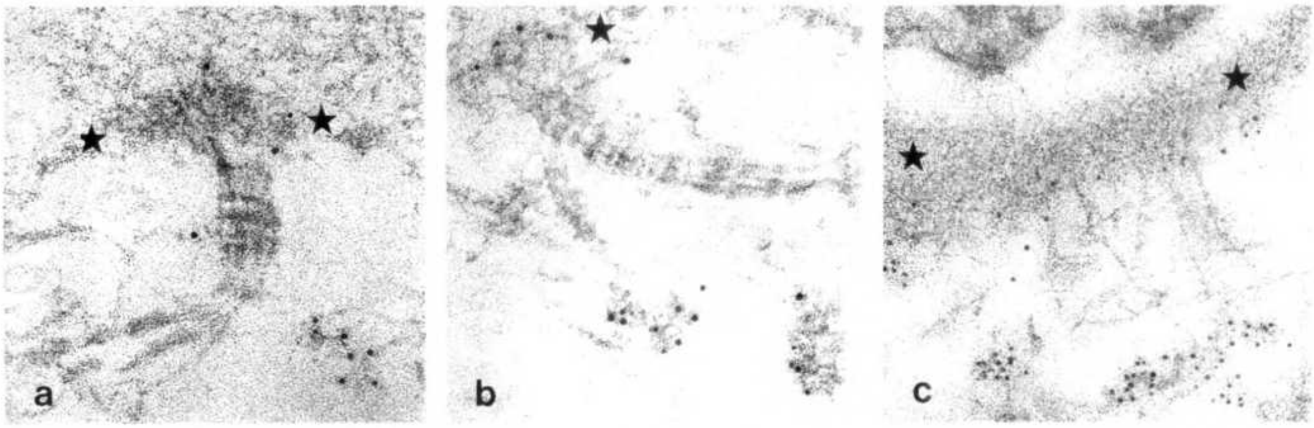

Figure 4. Staining pattern along the anchoring fibrils in the skin of Patient 2 exposed to anti-human IgA (a and b) as well as in normal human skin exposed to antibodies against the globular terminus of collagen VII (c). Note the similar distribution of gold particles in the upper (lamina densa) and lower (anchoring plaques) ends of anchoring fibrils in (a), (b) and (c). Stars indicate the lamina densa (a × 124,400; b × 124,400; c × 124,400).

was very similar to that seen in healthy skin stained by antibodies against the globular terminus of collagen VII (Fig. 4c).

There was no gold deposition in control skin samples exposed to anti-human IgM (Fig. 3d).

Normal human skin. In skin exposed to antibodies against the globular terminus of collagen VII, the anchoring plaques were stained, as was the lamina densa, but the latter more faintly (Figs 3c and 4c).

Discussion

In the present study we investigated two patients with LAD and found them to have two different patterns of antibody deposition in the diseased skin. In Patient 1 IgA was detected within the uppermost lamina lucida and was associated with the basal keratinocyte plasma membrane, in a manner very similar to the distribution of IgG in patients with bullous pemphigoid. There was no labelling either along the lower part of the lamina lucida, within the lamina densa, or along the anchoring fibrils. In Patient 2 we found a clearly different distribution of IgA, which was associated with the anchoring plaques. In addition, faint labelling along the lamina densa, on the upper parts of the anchoring fibrils was also observed. This staining pattern was ultrastructurally similar to the binding sites of antibodies against the NC_1 globular terminus of collagen VII within normal skin. Previous characterization of collagen VII suggested that this non-collagenous globular domain resides in the carboxyl terminus.[20,21] Recent cloning of the human type collagen VII, however, has proved that this globular domain corresponds to the amino-terminal segment of this protein.[22,23]

Yaoita and Katz described two types of ultrastructural distribution of IgA in patients with linear IgA deposition along the BMZ and also found binding of circulating antibodies in two different levels of the BMZ.[9,24] However, with the peroxidase method, the labelled structures could not be recognized in detail. Several subsequent studies, also using peroxidase techniques, repeatedly documented a lamina lucida or a sublamina densa location of IgA in LAD.[9–18] Dabrowski *et al.*,[10] and recently Prost *et al.*,[15] described a third group of LAD patients, characterized by IgA labelling both within the lamina lucida and sublamina densa regions of the BMZ. This staining was found in most of their patients. Furthermore, a LAD patient with IgA deposition within the lower part of the lamina lucida has also been reported.[14]

Until now, the target antigens of LAD have not been characterized. However, results of immunoblot studies recently published by Wojnarowska *et al.*[25] suggest that in patients with CBDC and adult LAD and sublamina densa deposition of IgA the target antigen is identical and is a dermal protein of 285 kDa molecular weight. These authors found this antigen to be different from collagen type VII under identical running conditions. Other authors have demonstrated an anti-collagen type VII specificity of IgA autoantibodies in some cases of LAD with sublamina densa immune deposits.[18] Recently, a 97-kDa protein was isolated from normal human dermal tissue in 1 M NaCl split skin and from both epidermal and dermal tissues in EDTA separated skin, and was identified as the target BMZ antigen in LAD.[26]

The fact that IgA and not IgG is deposited along the BMZ, was thought to be sufficient to establish LAD as a distinct entity among the blistering diseases. However, it may be difficult to be precise about the diagnosis when both IgA and IgG BMZ antibodies are present. The aetiology of LAD is unknown. Diseases preceding or accompanying LAD often involve mucosal surfaces or lymphoid organs, which are sites of IgA production and immunological function.[6,9,11,27–31] The many unresolved questions suggest a need to characterize the target antigens and to resolve their ultrastructural localizations.

Our observations show that two patients with different clinical pictures of LAD had ultrastructurally distinct IgA binding sites within the BMZ. One patient showed ultrastructural similarities with bullous pemphigoid, with IgA deposition within the uppermost lamina lucida underlying the basal keratinocyte plasma membrane. In the other IgA was detected along the lamina densa and within the anchoring plaques. The distribution of IgA in the second patient was similar to that of IgG in epidermolysis bullosa acquisita,[32] as well as to that of the globular terminus of collagen VII in normal skin. The results of the present study suggest that LAD may not be a single entity as is often suggested[4,17,33] but may comprise a group of diseases. Further studies involving a large number of patients will be necessary to confirm these findings.

Acknowledgments

Dr S.Kárpáti is supported by the Humbolt Foundation, Bonn, Germany. We are most grateful for the skilful technical assistance of Ingilt Pfab.

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British Journal of Dermatology (1992) 127, 119–121.

Failure to demonstrate the true resection margins of excised skin tumours: a case for routine marking

D.A.PATERSON, J.D.DAVIES* AND K.M.McLAREN

University Medical School, Edinburgh, U.K.

**Southmead Hospital, Bristol, U.K.*

Accepted for publication 30 March 1992

Summary A solution of silver nitrate in methanol was used to assess the demonstration of the true surgical excision margin at histology of 100 skin excisions for basal cell carcinoma. In 15% of cases the true margin was not presented and deeper sections were required. The findings highlight the need for routine marking of excision specimens of neoplastic skin lesions to prevent incorrect diagnosis of incomplete excision based on a false resection margin.

Adequate excision is central to the treatment of skin tumours, and elaborate methods exist for the full assessment of resection margins.[1,2] The pressure of specimen numbers in routine practice, however, dictates that transections or cruciate blocks, as detailed in standard trimming texts,[3,4] are more frequently used. The use of India ink to mark the under-surface of the excision biopsy is recommended to assist orientation during embedding and identification of the deep and lateral margins. In practice, however, in our department this is seldom carried out. Ideally during processing, tissue blocks are embedded and sectioned so that the surface to be visualized is orientated parallel to the microtome blade. This yields a section showing the true resection margins. If, however, there is angulation of the tissue during embedding and sectioning then the resulting section may reveal a false resection margin (Fig. 1). If the tissue blocks have not been marked, this may not be appreciated, and the result is an incorrect assessment of completeness of excision.

In a previous study[5] it was noted that marking resection margins with a solution of silver nitrate in methanol and water yielded brown silver salt deposition along the marked margin. This marking, although obvious at microscopy, was much less noticeable than India ink in tissue blocks. By using this method it is therefore possible to determine at microscopy if the true excision margin is presented to the reporting pathologist, while not making it obvious that the margins have been marked and thereby causing deviation from routine practice during embedding and sectioning. We report the use of this technique to determine whether failure to mark the margins of skin excision specimens may lead to incorrect assessment of completeness of excision.

Methods

One-hundred excision biopsies of lesions diagnosed clinically as basal cell carcinoma were studied. The specimens were fixed overnight in either 10% neutral-buffered formalin or Bouin's fixative. Prior to trimming, the under-surface of each specimen was painted with an 8% solution of silver nitrate in 50% water:methanol using a size 101 hair paint-brush. The specimen was blotted dry with a paper towel, and single or multiple blocks at 90° to the long axis were taken, to include the nearest margin of excision. The blocks were routinely processed and embedded in paraffin wax. Six-micron sections were then cut, and stained with Harris's haematoxylin and eosin.

At microscopy we recorded the histological diagnosis and the presence or absence of the true (pigmented) excision margin on the first slide examined. Where the true margin was not presented, successive deeper levels of up to 30 μ each were cut to visualize the true margin. The number of such levels was recorded, and the completeness of excision reported on the basis of the true margin. Where the true margin was not initially presented it was recorded whether reporting based on the false margin would have led to an incorrect assessment of completeness of excision.

Results

The histological diagnoses of the 100 cases are presented in Table 1. The true (pigmented) surgical excision margin was presented to the reporting pathologist in 85 cases. In the remaining 15 cases a false resection margin was presented and subsequent 30 μ deeper sections, up

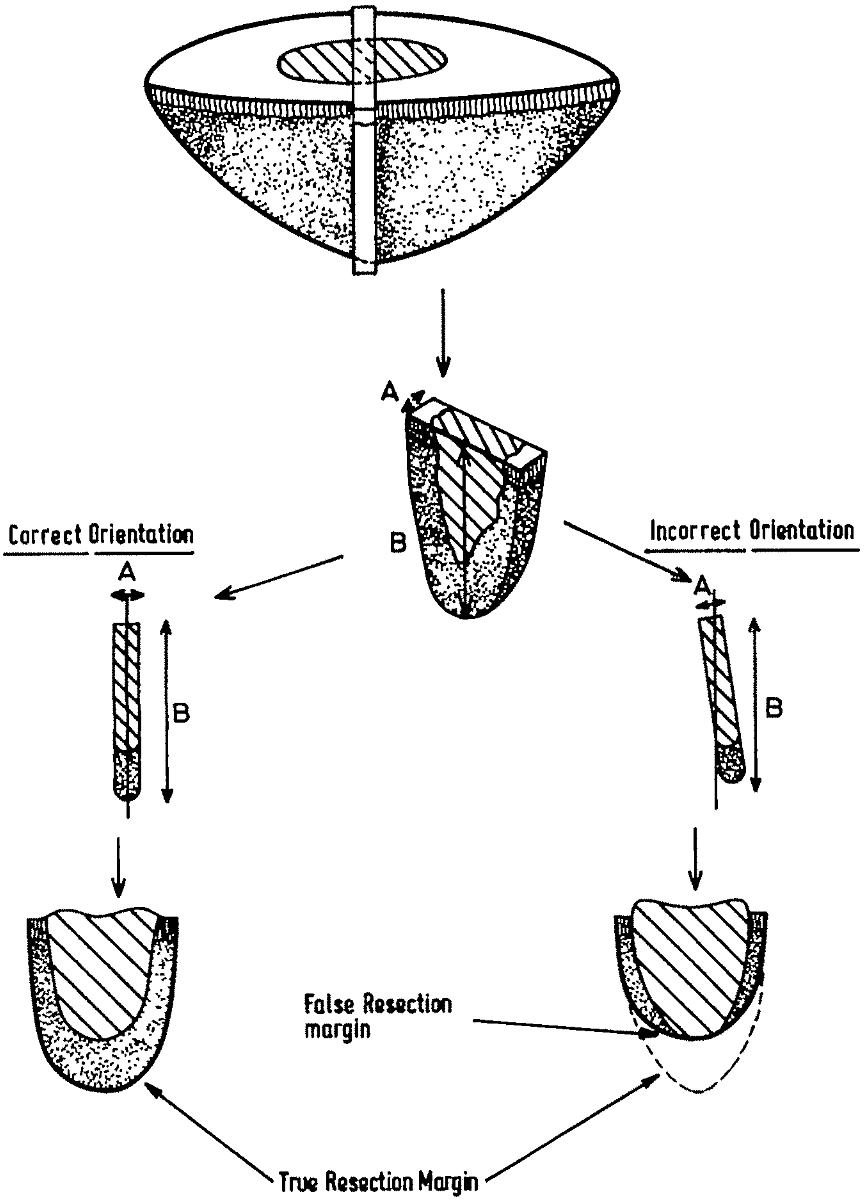

Figure 1. Diagram of skin excision specimen showing potential problem with incorrect orientation on sectioning.

Table 1. Histological diagnosis of 100 cases diagnosed clinically as basal cell carcinoma

Histological diagnosis	Number
Basal cell carcinoma	88
Squamous cell carcinoma	2
Bowens disease	3
Actinic keratosis	2
Seborrhoeic keratosis	4
Dermatofibroma	1
Total	100

Table 2. Number of 30 μ deeper sections required to view the true excision margin

Number of sections	Number
0	85
1	3
2	6
3	5
4	—
5	1
Total	100

to a maximum of five, were required to visualize the true resection margin (Table 2). Excision was considered incomplete based on the true resection margin in 14 cases. In none of the 15 cases where a false margin was initially presented would reporting based on the false margin have led to an incorrect report of incomplete excision.

Discussion

Although other features, such as recent haemorrhage, may be helpful in identifying the true excision margin at microscopy, marking with India ink or other pigments prior to trimming provides a more reliable assessment. The marking pigment needs to be readily visible in tissue blocks and India ink, despite its slow drying, is probably the most readily available and convenient. The silver nitrate solution was used for this study only, and because it is difficult to see during embedding it is not recommended for general use. Marking enables accurate reporting of completeness of excision given the limitations of the simple methods of specimen trimming in common use. It facilitates correct orientation of tissue blocks during embedding and sectioning, making measurements such as Breslow thickness of malignant melanoma,[6] more accurate.

While careful follow-up or cryocautery may be adequate management for basal cell carcinomas histologically reported as incompletely excised, for other neoplastic skin lesions such as squamous carcinoma and malignant melanoma incomplete excision is an indication for further surgery. In 15% of cases in this study the true surgical excision margin was not initially presented to the reporting pathologist. This did not result in a false diagnosis of incomplete excision only because of the wide excisions employed by our dermatologists. The potential for incorrect assessment is, however, real, especially in cases with relatively narrow margins of surgical excision. These findings support the routine marking of excision margins in cases of therapeutic excision for neoplastic skin lesions.

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British Journal of Dermatology (1992) **127**, 122–125.

Dynamic hepatic scintigraphy in the screening of psoriatic patients for methotrexate-induced hepatotoxicity

P.M.McHENRY, E.A.BINGHAM, M.E.CALLENDER,* P.B.DELVIN,† M.D.O'HARA,‡
W.R.FERGUSON,† J.D.LAIRD† AND D.BURROWS

*Departments of Dermatology, *Medicine, †Radiology and ‡Pathology, Royal Victoria Hospital, Belfast, U.K.*

Accepted for publication 22 January 1992

Summary We report the use of dynamic hepatic scintigraphy in the assessment of the hepatic status of psoriatic patients before and during methotrexate therapy. Eighty-seven paired dynamic scans and percutaneous liver biopsies were performed in 63 patients. The liver biopsies were graded according to Warin *et al.*[1] with fibrosis of grade 2 or worse being a strong indication for withdrawal of methotrexate. The sensitivity of dynamic hepatic scintigraphy in detecting fibrosis of grade 2 or worse was 83·3% and the specificity was 81·5%. The predictive value of a normal scan for fibrosis of grade 0–1 was high (98·5%) although the predictive value of an abnormal scan for fibrosis of grade 2 or worse was low (25%). Dynamic hepatic scintigraphy may therefore offer a means to reduce the number of liver biopsies necessary in patients receiving methotrexate for psoriasis.

Methotrexate is a well-established drug in the management of patients suffering from severe debilitating psoriasis. It is however, associated with several adverse effects, the most important of which are hepatic fibrosis and cirrhosis.[2–8]

Non-invasive methods such as the monitoring of serum liver enzyme levels,[3,9] ultrasound scanning,[10] magnetic resonance imaging[11,12] and static radionucleotide scanning[13] have all proved to be unreliable in detecting early hepatic changes. Liver biopsy remains the definitive investigation.

Percutaneous liver biopsy is not without risk and has a reported morbidity of up to 10%[14] and a mortality rate of 0·014%.[15] Therefore, any non-invasive investigation retaining the sensitivity of liver biopsy but avoiding its problems would represent a significant advance.

Dynamic hepatic scintigraphy (DHS), in the form introduced by Ferguson *et al.*[16] is a simple, non-invasive technique which provides consistent, reproducible results not influenced by observer variation. The liver has a dual blood supply, a high-pressure arterial system and the low-pressure system of portal venous blood flow. The low-pressure portal venous system is more susceptible to changes in hepatic structure such as those seen in damage due to methotrexate therapy. DHS aims to detect disturbances of liver function by the demonstration of a reduction in the portal venous contribution to total hepatic blood flow. This makes DHS a potentially more sensitive test of liver function than ultrasound scanning, magnetic resonance imaging and static radionucleotide scanning, which simply define hepatic morphology.

The aims of this study were firstly to determine the sensitivity and specificity of DHS in detecting significant liver fibrosis, and secondly to assess whether DHS might be used as a sensitive screening test to select those patients receiving methotrexate who require a liver biopsy.

Methods

It is our practice to perform a percutaneous liver biopsy on all patients prior to commencement of methotrexate therapy, and subsequently after every 1–1·5 g of methotrexate, or at approximately yearly intervals. Since 1981 we have carried out dynamic hepatic scintigraphy on patients requiring liver biopsy. For the purposes of this study we have compared results of the liver biopsy and DHS scanning when both have been carried out within a maximum interval of 4 weeks. Measurement of serum levels of γ-glutamyl transpeptidase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase and serum bilirubin was also undertaken within the same period.

Correspondence: Dr P.McHenry, Department of Dermatology, Western Infirmary, Glasgow G11 6NT, U.K.

Table 1. Grading of liver fibrosis (Warin *et al.*[1])

Grade		Fibrosis
0		None
1/2	Very mild	Just short of 1
1	Mild	Sparse intralobular
2	Moderate	Just bridging portal tracts
3	Severe	Bridging portal tracts

Liver biopsies were performed using a Tru-Cut needle, and were stained with haematoxylin and eosin, Foot's reticulin, Masson trichrome and Perl's Prussian blue reaction for iron and orcein. The biopsies were all reported independently by one pathologist and were classified according to Warin *et al.*[1] Of importance in methotrexate therapy is the degree of fibrosis (Table 1). As methotrexate may be continued in the presence of sparse intralobular fibrosis,[17] fibrosis of grade 2 or greater was considered an indication for methotrexate withdrawal and in this study was used to distinguish 'normal' from 'abnormal'.

DHS was performed in the manner of Ferguson *et al.*[16] Over an 80-s interval, following the rapid intravenous injection of a bolus of 111 MBq technetium99-labelled colloid into a peripheral vein, a sequence of images, showing initial uptake of colloid in the liver and spleen, is obtained using a large field of view gamma camera which is operated in conjunction with a nuclear medicine computer. By computer analysis two time–activity curves are derived which represent uptake of colloid by the liver and spleen over the period of data acquisition. The splenic uptake may be used to model the uptake of colloid reaching the liver by the arterial system. By normalizing the two curves at a point just before the onset of portal input of colloid, and subtracting the splenic from the hepatic curve, a third curve is obtained which represents the portal venous contribution to total hepatic uptake of colloid. A portal contribution of < 50% of total hepatic uptake of colloid at 30 s was regarded as abnormal. This cut-off point was chosen following studies on 50 patients with no liver disease (on the basis of biopsy or clinical follow-up), in whom the mean portal contribution was 64·4±11·7%.

Results

A total of 87 combined liver biopsies and DHS scans were performed on 63 patients (41 males, 22 females; mean age 52 years, range 21–84). For the 23 patients in this study who had paired liver biopsies and scans while receiving methotrexate, the mean cumulative dose of methotrexate was 1·5 g (range 60 mg–7 g) and the mean duration of therapy was 32 months (range 1 month–9 years). Forty-nine paired studies were performed prior to starting treatment with methotrexate and 38 during therapy.

Of the 87 paired liver biopsies and DHS studies, DHS was normal in 67 and abnormal in 20 (Table 2). One patient with a normal scan of 51% was found to have grade 2 fibrosis on liver biopsy (see below) whilst all other patients with normal scans had what were regarded as normal biopsies in that they were able to continue with methotrexate therapy (i.e. grade 0–1 fibrosis). Of the 20 abnormal scans, five patients had abnormal biopsies of grade 2 fibrosis or worse (Table 3) and 15 patients had normal biopsies of grade 0–1. The sensitivity of DHS in detecting fibrosis of grade 2 or worse is thus 83·3% (5/6) and the specificity is 81·5% (66/81). The predictive value of a normal scan for fibrosis of grade 0–1 is high at 98·5% (66/67) whilst that of an abnormal scan for grade 2 or worse is 25% (5/20).

No direct correlation was found between the cumulative methotrexate dose and the DHS result. This is not surprising, because although liver toxicity from methotrexate is felt to be related to the cumulative dosage, this relationship is by no means linear or predictable.

Although in this study an absolute value of 50% was used to distinguish normal from abnormal, if the cut-off point for abnormality had been 52% all of the patients with abnormal biopsies would have been picked up. A percentage reduction from a patient's own DHS baseline value may also be important. This is illustrated by the case of a male aged 66 who had an initial DHS value of 77% and a grade 1 liver biopsy. After 0·5 g of methotrexate taken over a period of 12 months, the scan result fell to 51% and liver biopsy showed grade 2 fibrosis. Subsequently, his methotrexate was discontinued and a repeat biopsy and scan 1 year later had returned to normal (biopsy = grade 1, scan = 74%).

Two further cases serve to illustrate the potential value of DHS. Firstly, a patient with an abnormal DHS

Table 2. Dynamic hepatic scintigraphy vs. liver biopsy results

	DHS		
Liver biopsy—Grade of fibrosis	Abnormal < 50%	Normal ≥ 50%	Mean DHS ± standard deviation (%)
2+3	5	1	46±5
0, 1/2, 1	15	66	65±18

Table 3. Details of patients with abnormal liver biopsies

Case	Age	Sex	? History of alcohol abuse	Cumulative methotrexate dosage (g)	DHS value (%)	Liver biopsy	
						Fibrosis grade	Cirrhosis
1	77	F	No	1·0	46	3	Present
2	47	F	Yes	—	48	3	Present
3	49	M	Yes	—	37	3	V. early
4	46	F	Yes	—	47·5	3	Early
5	42	M	Yes	—	45	3	Present
6	65	M	No	0·5	51	2	Absent
7	84	F	No	3·4	43	3	Present at post-mortem 5 months after DHS

(44%) had a normal liver biopsy. She did not receive methotrexate, but subsequently developed ascites and hepatic failure. Secondly, an 84-year-old woman received a total dose of methotrexate of 3·4 g over a period of 9 years. Two years after a normal DHS scan and liver biopsy, her DHS result became abnormal, with a portal contribution to hepatic blood flow of 43%. However, she refused a further biopsy, but because of the severity of her psoriasis methotrexate was continued. She died 5 months later from non-related causes but post-mortem findings included cirrhosis of the liver.

Monitoring of liver enzymes was found to be unhelpful in the detection of liver fibrosis. In almost 50% of the studies with normal liver biopsies, elevation of one or more liver enzymes was found, while in one patient with grade 3 fibrosis and cirrhosis the liver enzymes were entirely normal.

Discussion

In this study we have confirmed the hepatotoxicity of methotrexate. Three patients in our series had abnormal liver biopsies whilst receiving methotrexate. Only one patient had received a relatively high cumulative dose of methotrexate (3·5 g cf. 0·5 g and 1·0 g). As in other reports, we found measurement of liver enzymes an unreliable method of detecting liver disease.

DHS had an 83·3% sensitivity and 81·5% specificity in detecting liver fibrosis of grade 2 or worse. More importantly, the predictive value of a normal scan for fibrosis of grade 0–1 was high at 98·5%. Because it has been established that methotrexate therapy can be safely initiated or continued in patients with grade 0–1 fibrosis on liver biposy, but may be dangerous in those with grade 2 or 3 fibrosis, a normal DHS result may therefore be particularly useful in identifying patients who do not require a liver biopsy at that point in time. In this study population such a policy would have reduced the number of liver biopsies by 77%.

DHS is potentially a valuable tool in the detection of early hepatic damage due to methotrexate. Further studies with more abnormal liver biopsies are required to confirm our results.

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British Journal of Dermatology (1992) **127**, 126–130.

The phenotype of Darier's disease: penetrance and expressivity in adults and children

C.S.MUNRO
Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K

Accepted for publication 23 March 1992

Summary

The prevalence, pattern of inheritance, and phenotype of Darier's disease was examined in a cross-sectional study of cases in north-east England. Seventy-five cases, representing a local prevalence of at least 1 : 36,000, were identified and examined. Forty-two cases were known to dermatologists, and 33 were identified in the course of the study; 60 were adults (above the age of 20) and 15 were children. In nine families, 66 of 136 adults with an affected parent were known to be affected, indicating complete penetrance of an autosomal dominant gene. Eighteen individuals gave no family history, but seven of these were found to be members of affected kindreds and five obligate carriers examined all proved to have evidence of the disease. In other 'new' cases, including several whose parents were examined and confirmed to be normal, no relationship to other cases was found. These cases probably represent new mutations or non-paternity rather than incomplete penetrance.

All but one adult case had the typical rash, but it was milder in women; 16/18 adults who had not presented to dermatologists were female. Diagnostic nail lesions were found in 59 (99%), palmar pits and keratoses in 57 (95%), and acrokeratosis verruciformis in 44 (73%). In 15 young cases (aged 5–18) with one or more diagnostic features, the rash was found in only four (27%), nail lesions in nine (60%), palmar pits in eight (53%), and acrokeratoses in ten (67%). The minor lesions were often subtle but were more consistent early evidence of disease than the rash, and even in the absence of a rash gene penetrance may be complete by the age of 10.

Darier's disease (Darier–White disease, keratosis follicularis) is inherited as an autosomal dominant disorder, but cases with no family history are common, and represented a third of cases in one recent large series.[1] For this reason it has been assumed that penetrance is incomplete[2] or variable,[3] but a high rate of spontaneous mutation is suggested as an alternative source of 'new' cases.[4] As well as uncertain penetrance, there is some evidence that the gene may not always be fully expressed: relatives of cases displaying only minor manifestations such as nail changes,[5] or families in which the predominant lesions are the plane wart-like keratoses (acrokeratosis verruciformis, AKV),[6,7,8] are reported.

The possibility that the gene may be expressed partially or not at all is important for one approach to the study of the pathogenesis of Darier's disease, that of chromosomal mapping of the disease gene(s). It is essential for such linkage studies to be able to distinguish carriers of the gene from their normal kin, and further, as the rash does not usually appear until early adult life, to know from what age such a distinction can reliably be made. Moreover, a single clinical entity such as Darier's disease may be due to different genetic defects in different families, and variation in the phenotype may reflect such heterogeneity. In this study, therefore, a series of patients with Darier's disease from one area of England have been examined to establish, for adults and children, penetrance and expressivity of the gene, and variation in these between families. Cases who have no antecedent family history may suggest incomplete gene penetrance, and for these individuals, I have tried to establish whether other cases in the region are even distantly related.

Subjects

Patients with a clinical and/or histological diagnosis of Darier's disease were identified from diagnostic indices in four large dermatology departments together serving a population of 2,500,000 in Northumberland, Tyne and Wear, County Durham and Cleveland. As many of these patients as could be traced were contacted by letter, and asked to record their ancestry, sibships, and children, and any family history of the disease. In this way, a series of kindreds (designated A to M) was documented. Forty-five patients and 69 first-degree relatives agreed to be interviewed and examined; further information about relatives was obtained at interview. In addition, six deceased cases from affected families were known from medical records. In the cases examined, features considered to indicate the presence of the gene were the typical rash of Darier's disease, typical nail lesions, palmoplantar lesions (pits and keratoses), and plane wart-like lesions (acrokeratosis verruciformis, AKV). Palm prints were used to aid identification of mild palmar pitting. The severity of the rash was graded as 0 (none), 1 (mild, localized or sparse papular lesions), 2 (extensive papular or locally confluent lesions) or 3 (extensive confluent lesions). Thirteen patients taking etretinate were assigned severity grades according to pretreatment clinical notes.

Table 1. Gene penetrance in adults from families with Darier's disease

Family	Adults at risk*	Affected adults†	Normal adults	Inadequate information
A	42	18	18	6
B	27	13	11	3
D	4	1	3	—
E	4	2	1	1
F	29	15	6	8
H	5	5	—	—
I	4	2	2	—
L	5	2	3	—
M	16	8	5	3
Total	136	66	49	21

* Living and deceased individuals with an affected parent, over the age of 20, and in current generations.

† Including six known cases now deceased.

Results

Seventy-nine cases of Darier's disease were identified and examined. Four patients with naevoid lesions were excluded from further analysis. Of the remaining 75, 42 had already presented to dermatologists and 33 were identified amongst relatives during the study. Fifteen cases were aged under 20 years and 60 were adults. Thirteen families with two or more affected members were identified. Seventy cases lived within the area under study, giving a local gene prevalence of 1 : 36,000; this is a slight underestimate because other reputed and known cases did not respond to requests for information.

Penetrance in families

Penetrance was estimated in members of current generations in nine families. Among 136 adults in whom parental involvement was known or could be deduced, information was available on 115. Sources of this information were clinical examination (78), medical records of deceased cases (6) or, because in most cases the problem was recognized by the families themselves, clear and corroborated accounts by first-degree relatives (31). Sixty-six (59%) of these 115 individuals were affected. This is more than half, but because 21 other members for whom information was inadequate are less likely to be affected, the figures are consistent with complete penetrance of an autosomal dominant gene (Table 1). There was no evidence that any offspring of unaffected family members had the disease. Eight such offspring were examined, in three cases because they were said to have skin lesions, but only eczema or acne were found.

Possible incomplete penetrance

Eighteen patients, 43% of the 42 cases originally known to dermatologists, gave no history at presentation of Darier's disease in parents or siblings. Detailed pedigrees were obtained in 14 of these cases whose families lived in the region. As a result seven were revealed to be relatives of known cases: two were estranged siblings whose parents were untraceable and the other five were related to known kindreds. Each of the latter had a living mother who was an obligate carrier. When traced, all five mothers were found to have evidence of disease. One did not have a rash, but had other lesions (see below) and the other four had a rash and two or three other features. In all these cases the rash was mild and the diagnosis had not been made prior to the study. In the remaining cases without antecedent family history, no link to other patients was established by documenting their relatives. Parents of six cases with no known antecedent family history or relationship to other cases agreed to be examined. In four, only one parent was living, in a fifth only the mother was seen, and in one case both parents were examined. None had any evidence of Darier's disease. Nevertheless, eight of 14 adult offspring, and one of two younger children, of patients without antecedent history were known to be affected.

Table 2. Features of Darier's disease in adults

Family	Cases examined	M:F	Newly diagnosed	Rash	Nail lesions	Palmar lesions	AKV
A	15	6:9	6	14	15	14	10
B	11	2:9	7	11	11	11	8
F	6	4:2	1	6	6	5	6
H	6	3:3	1	6	6	6	6
M	5	1:4	3	5	4	5	2
Other families	10	5:5	—	10	10	9	7
'Lone' cases	7	4:3	—	7	7	7	5
All cases	60	25:35	18	59 (99%)	59 (99%)	57 (95%)	44 (73%)

Gene expression in adults

In adults, a diagnostic rash was present in 59/60 cases who had evidence of the disease gene. The exception was a 75-year-old woman, an obligate carrier, who had palmar keratoses, AKV, and notched dystrophic nails, but who denied any history of skin lesions. Diagnostic nail and palmar lesions were almost equally as common as the rash, but AKV, sometimes only present as multiple minute acanthomas of the dorsum of the hand and volar forearm,[9] was found in 73% (Table 2). This pattern was the same in all families. Notably, etretinate therapy did not appear to suppress any of the minor features. The severity of the rash varied between cases within families, gender being the most important determinant; 18/35 women had mild or no skin disease compared with 7/25 men. Of the 18 adults who had not presented to dermatologists, only two were male. The median age of onset of the rash, according to the patients, was 21 years (range 7–64), and that of the nail lesions, 14 (6–32). Once present, there was no evidence of increasing severity of the rash with increasing age. There were no gross differences in the distribution of the rash between families, except that both the members of Family G seen, who were related only distantly, had flexural disease of a severity not seen in other cases.

Other features

There were no other frequent cutaneous features, although two affected sisters in Family B had a moderate ichthyosis. Consistent neurological or intellectual defects were not found. In two cases mental subnormality was clinically notable, but the only severe case was a woman with severe retardation requiring institutional care, and epilepsy of unknown cause, since birth. Her brother who also had Darier's disease was of normal intellect, and several cases in other families had skilled or professional employment. An affected mother and child of four cases in one family had also been treated for fits, as had two affected sisters and the daughter of one (all deceased) of 15 cases in another family. Amongst a family of 13 cases, a patient and her late mother had required admission to psychiatric hospitals many years previously: details were not available.

Gene expression in childhood

Thirty-six children (age <20) of cases of Darier's disease were examined for features of the condition. Evidence of gene expression was found in 15. None of eight children aged less than 5 years had definite diagnostic features, although a 3-year-old had possible early nail changes.

Table 3. Signs of the Darier's disease gene in 15 cases aged under 20

Age	Sex	Rash	Nail lesions	Palmar pits	AKV
5	F	–	–	–	+
5	M	–	–	+	+
6	F	–	+	+	–
7	F	–	+	–	+
8	F	+	+	–	+
8	M	–	–	+	+
9	M	–	+	–	+
9	F	+	–	+	–
11	F	–	–	+	+
12	M	–	+	+	+
13	M	+	–	+	+
14	M	–	+	+	+
14	F	–	+	–	–
17	M	+	–	+	–
18	M	–	+	–	–
	8M:7F	4 (27%)	8 (53%)	9 (60%)	10 (67%)

Eight of 13 aged 5–9, five of eight aged 10–14, and two of seven aged 15–19 had diagnostic features (shown in Table 3). Only four young cases had the typical rash when first seen. AKV, often represented by multiple minute acanthomas, and nail changes, were the most easily recognized early evidence of the presence of the gene. Palmar pits, while often present, were scanty and difficult to see except on palm prints.

Discussion

The prevalence of the Darier's disease gene in north-east England (>1:36,000) is greater than the previous estimated frequency of the disease in Oxford (1:50,000)[5] or Denmark (1:100,000).[6] This high prevalence is in part due to several large undispersed kindreds. Such families, in each of which affected members must share a single defective gene, are of enormous potential value in molecular genetic studies which aim to identify a pathogenic defect in known or new genes. However, for the purpose of mapping the gene(s), the penetrance and expressivity must be well understood. In this study, I have tried to determine whether the Darier's disease gene ever fails to be expressed, or is expressed only as a *forme fruste*.

It is difficult to prove the negative, that non-penetrance in adults never happens, but penetrance in the kindreds documented here is complete and there is no contrary evidence in earlier studies of large[6,10] or small[11] families. However, the frequent cases with no antecendent history still raise the possibility that in some families penetrance might be incomplete. About half the present subjects who thought they had no family history were related to known cases and obligate carriers examined were all affected. Gene expression never 'skipped a generation'. Conversely, where no connection to any other known case could be made, examined parents were unaffected, but the 'new' gene defects were transmitted to the next generation as an autosomal dominant. Incomplete penetrance is thus unlikely to explain new cases. Previous authors have alternatively concluded that new cases have arisen because of spontaneous mutation. A third possible explanation, non-paternity, has been ignored. The frequency of this in the general population is uncertain[12] but its omission from consideration is surprising in view of the social deviancy which was in the past considered a common feature of the disorder.[13]

While penetrance is complete in adults, expressivity varies, particularly in the severity of the rash. Women have less severe disease than men and the rash occasionally escapes medical notice altogether. Minor features of gene expression, the palmar and nail lesions, were none the less almost universal. AKV was least common, but in some cases may have been present earlier in life. There was no evidence of remission of nail or palmar lesions.

Despite claims that mental illness or insufficiency are typical features of the disease,[6,7,11,13] in the present study group this was not a notable feature. It is not possible to conclude that the disease gene is never responsible for mental subnormality or illness, or perhaps epilepsy, but such cases were exceptional and not consistent within families. For the British population at least,[1] the prejudicial stereotype of sufferers as subnormal, subfertile sociopaths should be rejected.

In children carrying the Darier's disease gene, nail changes are one of the first expressions, and precede the appearance of the rash. Retrospective accounts of adults also indicate that the nail changes occur before the rash, although it seems likely that both are present in mild degree before they are recognized by the patient. AKV, too, precedes the rash,[9] and very numerous 'plane warts' in a child should raise at least the suspicion of Darier's disease. The present, cross-sectional, study confirms that the early signs, common before the age of 10 and identifiable as early as the age of 5, include the multiple minute acanthomas which appear to evolve into AKV. Single or sporadic nail lesions may also be present at these ages, but the sparseness of palmar pits makes them less easy to detect. Despite an impression that penetrance is high even by the age of 10, the inconsistent pattern of these sometimes subtle early features means that while the disease can be identified early, it remains difficult to be certain that a child is not affected before mid-to-late teens.

With the possible exception of severe flexural disease, there was no evidence in this study of heterogeneity of phenotype between families which might suggest more than one disease gene. The population was from a limited geographical area, and many may share a forgotten progenitor and thus a single gene defect, but as a group they have a similar pattern of phenotype to 145 different British cases examined by Burge and Wilkinson.[1] The claim that mental subnormality is common in Darier's disease in Denmark[6] might indicate different gene(s) in that country. Other disorders suggested as variant forms of Darier's disease include acrokeratosis verruciformis of Hopf.[8,14] In the present group the rash of Darier's disease was present in all but one adult patient, in contrast with the reported family of whom only two out of 12 affected by AKV had the rash,[8] which raises the possibility that this family had a genetically distinct disorder of similar phenotype.

Nevertheless, genodermatoses which display phenotypic overlap with Darier's disease, such as acrokeratosis verruciformis of Hopf[8,14,15] and perhaps Hailey–Hailey disease,[16] may prove to be due to pathogenetically related defects. The availability of larger numbers of cases of Darier's disease makes it a better first choice for study. Identification of the defective gene(s) of Darier's disease by linkage analysis with chromosomal markers[17] or candidate genes is made considerably easier by the existence of large families sharing a single defect, and the knowledge that adult carriers of the gene can confidently be distinguished from their unaffected siblings.

Acknowledgements

I am grateful to the consultant dermatologists of the Northern Region, all of whom have allowed me to study patients in their care.

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British Journal of Dermatology (1992) 127, 131–137.

The influence of daily dish-washing with synthetic detergent on human skin

G.KLEIN, G.GRUBAUER AND P.FRITSCH
Department of Dermatology, University of Innsbruck, Austria

Accepted for publication 9 March 1992

Summary The effects of regular dish-washing on the stratum corneum barrier function, as determined by transepidermal water loss (TEWL), and objective and subjective skin parameters, were investigated in a 'use test' performed by 18 healthy volunteers. Hands were soaked in a 0·05% solution of a commercial dish-washing product (three times/day, for 15 min, at 37°C) over a period of 3 weeks; one hand was unprotected and the other was protected with a commercial rubber glove. TEWL increased in 13/18 volunteers by more than 25% above baseline on exposed hands within the first 2 weeks of the study. Objective skin signs (erythema, scaling, fissures) and subjective symptoms (itching, dryness, smarting) occurred almost exclusively in subjects with substantial TEWL increases and were most prominent 1–2 weeks following peak TEWL values. There was a highly significant correlation between cumulative relative symptom scores and TEWL changes. TEWL increase and symptom scores were not correlated with a history of inhalant allergy and/or elevated serum IgE levels.

Three volunteers, who had shown the highest increase of TEWL values and the most intense clinical reactions to the detergent were subjected to a control experiment in which one hand was soaked in warm tap-water following the same experimental protocol. No significant effects on TEWL values or skin symptoms were observed. We conclude that regular exposure to low concentrations of detergents as used for dish-washing is capable of inducing skin lesions in a substantial proportion of individuals.

Public opinion holds that regular use of washing-up liquids and household detergents is harmful to the skin by causing hand eczema and a variety of other skin diseases, which are, in fact, unrelated to detergents.[1] In dermatological textbooks, the role of soaps and detergents in hand eczema is infrequently discussed or considered controversial. Fisher[2] states that, 'although it is not certain that soaps and detergents play a major role in hand eczema, contact with them should be minimized, especially in atopic persons'. In classic clinical studies,[3,4] no negative influence of detergent use was found on normal skin or the healing of skin diseases in the vast majority of volunteers. Suskind *et al.*[4] concluded that, 'soap or detergent immersion alone in household usage may not be a significant primary provoking stimulus or aggravating factor in eczematous problems found in housewives ...'.

In contrast, several recent studies have demonstrated that detergents may cause disturbances of the skin. Detergents in washing-up liquids can induce rough, dermatitic skin,[5] increase stratum corneum turnover,[6] and have irritating potencies depending on the chemical structure of the detergent.[7] These changes correlate with a significant increase in transepidermal water loss (TEWL), which has been shown to be a more sensitive marker of epidermal changes than visual scoring.[6,7] Furthermore, susceptibility to irritants has been shown to be greater in persons with higher baseline transepidermal water loss,[8] may decrease with age[9] but does not seem to be correlated with sex.[10]

A possible causative role for detergents in chronic hand eczema ('housewife's eczema') is nevertheless hotly disputed by many, including the manufacturers of detergents, who prefer to view this condition as a multifactorial disease in which detergents play a negligible role. In fact, the evidence linking detergent use in everyday life to hand eczema is incomplete. In the studies cited above, detergents were applied in considerably higher concentrations than normally used for washing purposes,[6,10] and sometimes under occlusion.[7]

Furthermore, commercial washing-up liquids contain many ingredients other than detergents, which may modify their effects on the skin. Finally, all recent studies have been performed on forearm or back skin instead of

Correspondence: Dr G.Klein, Universitatsklinik für Dermatologie und Venerologie, A-6020 Innsbruck, Anichstraße 35 Austria.

the hands, and thus disregard possible important regional differences in the response to irritants.

In this study, we attempted to determine the effects of a commercial dish-washing product on the hand skin in a controlled 'use test' simulating the conditions prevailing in an average Austrian household.

Methods

Subjects

Twenty healthy volunteers (13 women, seven men; aged 19–65 years) participated in the study. None had a history of hand eczema or skin symptoms at the beginning of the study. Two subjects defaulted for personal reasons unrelated to the study. Of the remaining 18, nine had been regularly using washing-up liquids prior to the study, four occasionally, and the remaining five rarely or never. Details of history for atopy and nickel allergy are given in Table 1.

Experimental design

The volunteers were asked to soak their hands three times a day for 15 min in 10 l of warm water (approximately 37°C) to which 5 ml of commercial washing-up liquid (Sunlicht ®, a very popular brand in Austria) was added, resulting in a 0·05% solution. The exact composition of this product could not be obtained from the manufacturer; however, information was provided that a washing-up liquid of this kind typically contains 20–40% of an anionic detergent. During immersion, one hand was unprotected (exposed hand; EH), and the other was protected with a commercial rubber glove (control hand; CH). This procedure was carried out for 21 consecutive days. No emollient was allowed during the entire study. Examinations were performed on days 0, 8, 15 and 22, at least 2 h after the last soak or other contact of the hands with water. Experiments were performed over a 6-week period in April and May 1990.

After an interval of 6 weeks, the three subjects who had exhibited the clinically most severe reactions were asked to take part in a control experiment. Following the same protocol, one hand was soaked in hand-warm water without detergent and the other hand was left untreated. All three subjects were completely free of symptoms at the beginning of the control experiment.

Table 1. Peak relative TEWL on days 8 or 15 cannot be predicted by age, sex, presence of atopy or use of detergents prior to the study, but show a strong correlation with CRSS ($\rho_{corr}=0{\cdot}63$ for $CRSS_{obj}$ and $\rho_{corr}=0{\cdot}66$ for $CRSS_{subj}$, $P<0{\cdot}01$, Spearman rank correlation)

							CRSS			
No.	Age	Sex	History	IgE U/ml	SX-1	Prior use of detergent	obj	subj	total	Peak relative TEWL*
1	65	F	Neg.	848	Neg.	Regular	12	25	37	7·3
2	37	F	inh.all.+ni.all.	53	Pos.	Regular	0	29	29	4·3
3	26	F	ni.all.	31	Neg.	Regular	21	22	43	7·1
4	29	M	Neg.	23	Neg.	Occasional	6	37	43	3·5
5	32	F	Dry skin	18	Neg.	Regular	36	25	61	14·3
6	30	F	ni.all.	75	Neg.	Regular	0	11	11	4·7
7	23	F	inh.all.+ni.all.	26	Neg.	Regular	24	31	55	25·1
8	37	M	Neg.	12	Neg.	Occasional	3	14	17	11·3
9	18	M	Dry skin	10	Neg	Never	0	0	0	3·1
10	25	M	Neg.	75	Neg.	Occasional	0	0	0	1·4
11	19	F	Dry skin	21	Neg.	Never	6	6	12	4·4
12	33	F	inh.all.	59	Pos.	Regular	6	17	23	5·5
13	19	M	Neg.	215	Pos.	Never	0	3	3	4·2
14	23	F	Neg.	17	Neg.	Never	0	2	2	3·9
15	22	M	Neg.	ND	ND	Regular	0	0	0	−1·8
16	32	F	ni.all.	25	Neg.	Occasional	0	0	0	3·5
17	44	F	inh.all.+ni.all.	601	Pos.	Regular	3	10	13	7·1
18	19	M	inh.all.	75	Pos.	Never	0	5	5	0·2

ni.all., contact allergy to metals.

inh.all., rhinitis and/or asthma of presumably allergic origin.

* Highest individual relative TEWL value, measured on days 8 or 15 in all volunteers (g/m^2 h).

Clinical evaluation

Subjective evaluation. Subjects were asked by investigator 1 (GG) to grade the subjective symptoms (itch, dryness, smarting) of both their hands (EH and CH) on a 10-degree visual scale prior to objective evaluation.

Objective evaluation. Objective clinical signs on the dorsa of both EH and CH were recorded by investigator 2 (GK), who was blinded to which hand had been in detergent, and graded according to Frosch and Kligman.[11] (i) Erythema = slight redness, spotty or diffuse (+); moderate redness (+ +); intense redness (+ + +); fiery red with oedema (+ + + +). (ii) Scaling = fine (+); moderate (+ +); severe with large flakes (+ + +). (iii) Fissures = fine cracks (+); single or multiple broader fissures (+ +); wide cracks with haemorrhage or exudation (+ + +).

Calculation of symptom scores

Symptom scores were calculated for objective clinical signs as well as subjective clinical parameters on EH and CH, respectively; taking the different scales for subjective symptoms and objective clinical signs into account, a (+) reaction in each of the objective clinical signs category was counted as 3 points, whereas 1 point on the visual scale was counted as 1. Values at the different time points during the study (days 8, 15, 22) were corrected by subtracting baseline values (day 0) and termed 'absolute symptom scores'. Absolute symptom scores of EH minus those of CH were defined as 'relative symptom scores' (RSS). RSS, again, were calculated for objective clinical signs ($\mathrm{RSS_{obj}}$) and subjective symptoms ($\mathrm{RSS_{subj}}$) for each time point (days 8, 15 and 22) and for the entire study period ('cumulative relative symptom scores', $\mathrm{CRSS_{obj}}/\mathrm{CRSS_{subj}}$). Finally $\mathrm{CRSS_{obj}}$ and $\mathrm{CRSS_{subj}}$ were summated to give a 'total cumulative relative symptom score' ($\mathrm{CRSS_{total}}$). The highest possible RSS (obj and subj) was thus 62 points for any time point in a given volunteer, and the highest possible CRSS total was 186 points.

Measurements of transepidermal water loss

TEWL was measured with a ServoMed Evaporimeter (ServoMed AB, Vallimgby–Stockholm, Sweden) at 22 ± 1°C and a relative humidity of 45 ± 5%. To ascertain precision of the evaporimeter a 10-cm Petri dish, filled with 10 ml of water and covered with an Op-site® membrane, was used as an external control.[12] The mean TEWL of nine different measurements on 9 different days was $7{\cdot}3 \pm 0{\cdot}74$ g/m^2 h. Each volunteer was tested after an acclimatization period of 15 min, the probe being applied perpendicular to the skin surface. After 45 s the filters (10 and 20 s) were switched on to dampen fluctuations. After switching on the filters, TEWL was measured for another 30 s, and only stable TEWL values were recorded (variation allowed was $0{\cdot}5$ g/m^2 h).

Evaluation of TEWL data

Individual TEWL values were considered 'substantially' increased if exceeding baseline (day 0) values of the respective test subject by more than 25%. In addition, mean TEWL values of all 18 test subjects were calculated for EH and CH at all time points, before and after correction for individual baseline values. Finally, individual and mean relative TEWL values ($[\mathrm{EH_{day\ x}} - \mathrm{EH_{day\ 0}}] - [\mathrm{CH_{day\ x}} - \mathrm{CH_{day\ 0}}]$) were determined for all time points.

Laboratory tests

Total serum IgE levels (Pharmacia IgE RIA®) and allergen-specific IgE to different inhalent allergens (multi SX-1 RAST®/Phadiatop®) were determined in all volunteers.

Statistical analysis

The Friedman Test and Wilcoxon's signed-rank test were used to compare absolute and relative TEWL values as well as RSS (objective and subjective) before, and at the various time points of the study. Spearman rank correlation was used to test for associations between RSS and relative TEWL values. Calculations were performed with 'Stat View SE + Graphics' on a Macintosh SE. Contingency tables were analysed with Fisher's exact test in 'Statgraph' on an IBM PC.

Results

IgE levels, CRSS and peak relative TEWL values for each volunteer during the study are summarized in Table 1. Total IgE was elevated (> 120 U/ml) in three subjects, and SX1 was positive in five volunteers, giving a total of six who were considered to be atopic. Only one of these had a history of atopic dermatitis (although symptom-free at the beginning of the study). Four had a history of rhinitis and/or asthma.

Clinical parameters

Only four subjects showed no change of subjective symptoms or objective clinical signs throughout the study. In all, IgE levels and SX-1 tests were normal; a history of 'dry skin' and of allergy to metals was given by one volunteer each.

Objective symptoms. Clinical signs became manifest in the EH of 10/18 subjects: erythema in five, scaling in 10 and fissures in two at days 15 and/or 22. Of these 10 subjects, five had a history of allergic rhinitis and/or asthma, 'dry skin' or allergy to nickel, and three had elevated IgE and/or a positive SX-1 test. The CH, in contrast, exhibited slight erythema in two and minor scaling in five subjects, either at day 8 , 15 and/or 22.

Subjective symptoms. Symptoms from the EH were reported by 14/18 subjects at days 8, 15 and/or 22: itching (10), dryness (12) and smarting (13). Twelve of these 14 had symptoms from their CH also, although these were mild: itching (6), dryness (12) and smarting (11). Of these 14 subjects, nine had a history of allergic rhinitis and/or asthma, 'dry skin' or allergy to nickel, and six had elevated IgE and/or a positive SX-1 multitest.

Relative symptom scores. In 9/18 subjects there was a distinct increase of $\mathrm{RSS}_{\mathrm{obj}}$ during the study, and in four cases these changes were marked. The changes were most pronounced on day 15 in six subjects and on day 22 in three. Eleven out of 18 subjects exhibited remarkable increases of their $\mathrm{RSS}_{\mathrm{subj}}$, the highest values being observed on day 8 in one and on days 15 and 22 in five subjects each. An additional three volunteers showed minor changes of $\mathrm{RSS}_{\mathrm{subj}}$. Highest objective and subjective RSS were observed either 1 or 2 weeks following peak TEWL values of the respective individual; in only one volunteer, peak TEWL values coincided with the highest RSS (day 8). The CRSS (obj, subj, total) for each volunteer are given in Table 1.

Transepidermal water loss (TEWL)

At the beginning of the study, mean TEWL values were equal for EH and CH ($11\cdot 79 \pm 4\cdot 8$ vs. $11\cdot 86 \pm 4\cdot 1$). TEWL of the EH increased substantially (> 25% above baseline) in 13/18 subjects (72%) during the first 2 weeks, and slightly decreased thereafter; in the remaining five, increases of TEWL were minimal or absent. Mean TEWL values of EH increased during the study and were significantly higher than baseline on days 8 and 15 (Table 2). In CH, in contrast, TEWL increased substantially (> 25%) in only 2/18 subjects; mean TEWL values of CH did not change significantly throughout the study. After subtraction of baseline values for each subject, mean TEWL values were significantly higher on EH than on CH, both on days 8 and 15 ($P<0\cdot 025$, Wilcoxon signed-rank test; Fig. 1). Furthermore, relative TEWL values on day 8 were highly correlated with the presence of objective clinical signs and complaints about subjective symptoms on day 15 ($\mathrm{RSS}_{\mathrm{obj}}$: $\rho_{\mathrm{corr}}=0\cdot 7$, $P<0\cdot 005$; $\mathrm{RSS}_{\mathrm{subj}}$: $\rho_{\mathrm{corr}}=0\cdot 54$, $P<0\cdot 03$; Spearman rank correlation), while there was no statistically significant correlation between relative TEWL values and RSS on other days. However, individual peak relative TEWL values (either measured on day 8 or 15) were highly correlated with the individual CRSS (Table 1; $\rho_{\mathrm{corr}}=0\cdot 63$ for $\mathrm{CRSS}_{\mathrm{obj}}$ and $\rho_{\mathrm{corr}}=0\cdot 66$ for $\mathrm{CRSS}_{\mathrm{subj}}$, $P<0\cdot 01$).

Table 2. Mean TEWL values ($\mathrm{g/m^2\,h} \pm \mathrm{SD}$) during study. These values on EH increased during the study and were significantly higher than on day 0 ($\chi^2 = 10\cdot 73$, $P=0\cdot 013$; Friedman test), whereas there was no significant change of TEWL values on CH ($\chi^2=1\cdot 17$, $P=0\cdot 76$; Friedman test)

Day	Exposed	Control
0	$11\cdot 79 \pm 4\cdot 8$	$11\cdot 86 \pm 4\cdot 1$
8	$15\cdot 13 \pm 7\cdot 9$*	$12\cdot 39 \pm 4\cdot 5$ NS
15	$17\cdot 14 \pm 6\cdot 9$*	$12\cdot 75 \pm 3\cdot 9$ NS
22	$15\cdot 05 \pm 7\cdot 1$ NS	$12\cdot 27 \pm 4\cdot 5$ NS

Differences to day 0 (Wilcoxon signed rank test):
* = $P \leqslant 0\cdot 01$; NS, not significant.

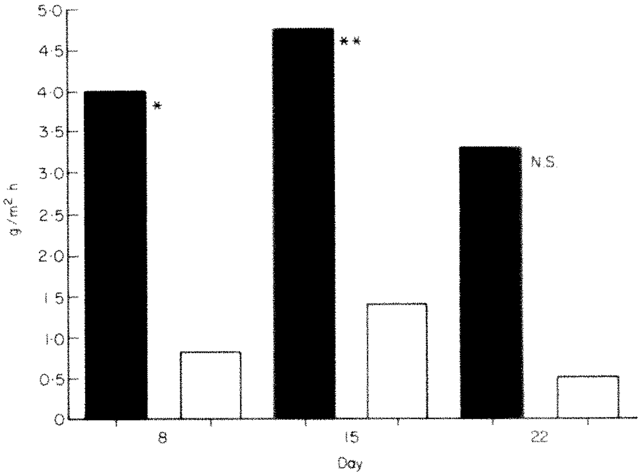

Figure 1. TEWL values at different time points during the study corrected for baseline. Differences between EH (■) and CH (□) are significant on day 8 (*$P=0\cdot 01$) and day 15 (**$P<0\cdot 05$).

Table 3. Relation of elevated IgE and relative symptom scores to TEWL increase. Note the strong trend between the occurrence of elevated IgE as well as positive objective and subjective RSS and substantial TEWL increase (>25% above baseline) on EH

	TEWL increase >25% of baseline		
	yes	no	Significance*
Total IgE >120 U/ml†			
Yes	5	1	NS
No	8	3	
Objective RSS			
Pos.	8	1	NS
Neg.	5	4	
Subjective symptoms			
Pos.	11	1	$P \leqslant 0{\cdot}05$
Neg.	2	2	

* Fisher's exact test.

† One serum not investigated.

Elevated IgE as well as objective clinical signs and subjective symptoms on EH were almost exclusively found in subjects with substantial increases of TEWL; nevertheless, this relationship was statistically significant at a 95% confidence limit only for subjective symptoms (Table 3). Mean relative TEWL values were consistently higher in individuals with objective clinical signs than in those without (days 8, 15 and 22); this trend was statistically significant only on day 8 (5·6 vs. 1·5 g/m^2 h; $P \leqslant 0{\cdot}025$).

In the control experiment, the three subjects who had exhibited the most severe reactions to detergent exposure, did not develop objective or subjective symptoms when exposed to plain water, and there was no substantial increase of TEWL values (Fig. 2a and b).

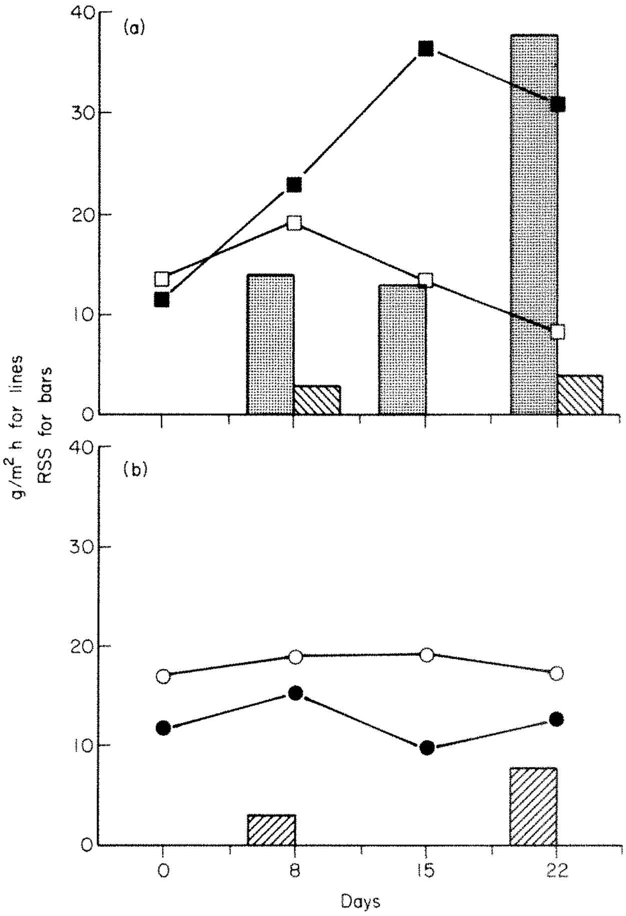

Figure 2. (a) Changes of TEWL (■, EH; □, CH) and RSS (▤, EH; ▨, CH) during exposure to a 0·05% solution of a commercial washing up liquid (three times/day for 3 weeks) in volunteer 7. Recordings were performed at least 2 h after the previous soak. Note the significant increase of TEWL on EH during the first 2 weeks of the study and of RSS in the third week, whereas changes on CH (protected with a commercial rubber glove) were only minor. (b) Changes of TEWL and RSS during the control experiment performed 6 weeks after the initial experiment and complete clearing of skin signs in volunteer 7. Recordings were performed at least 2 h after the last soak in warm tap-water without detergent. No substantial change of TEWL was observed on the EH (●) and the unexposed CH (○). Increased of RSS on EH (▨) was only minor. RSS did not change on unexposed CH.

Discussion

Adverse reactions of the skin to detergents have been shown to depend on the chemical nature and the purity of the surfactant,[7,13] its concentration,[9,14-16] type of application (open/under occlusion),[17] and frequency and duration of exposure.[8,17] We were informed by the manufacturer that the washing-up liquid used in this study contained 20–40% of an anionic detergent; from this we can calculate a final concentration of detergent of 0·01–0·02%. This is very low when compared with the concentrations used in previous studies (0·25–7·5% detergent,[5-9,13-16] usually sodium lauryl sulphate, with or without occlusion). It was therefore surprising that even this low concentration proved capable of inducing significant changes in the skin of a considerable proportion of volunteers. Objective clinical signs, which could be attributed to detergent exposure by positive RSS$_{\mathrm{obj}}$, were found on 9/18 (50%) detergent exposed hands, and 14/18 volunteers (78%) reported subjective symptoms such as itching, dryness and smarting of the skin and positive RSS$_{\mathrm{subj}}$. Cumulative RSS for both objective signs and subjective symptoms in the individual volunteers were highly correlated with increased TEWL ($P = 0{\cdot}015$). Thirteen out of 18 volunteers (72%) had significantly higher TEWL values after detergent use compared with both baseline values and with values on the protected hand. Thus even four persons without appreciable skin signs developed a significant subclinical alteration of the epidermal barrier function. Furthermore, in all but one individual, the highest RSS were recorded 8–15 days after the peak relative TEWL values. These results are in agreement with published data[6,7] indicating that increase of TEWL is a more sensitive marker of epidermal barrier impairment than symptom scores.

In our study the mean peak TEWL values occurred at day 8 or 15 and were 1·5 times (range 0·9–3·1) higher than the mean baseline TEWL levels ($P=0{\cdot}01$). This increase is in the same range as reported in the literature for several different surfactants tested in a ×10 higher concentration.[7,10] Higher increases in mean TEWL values have only been reported for sodium lauryl sulphate used in ×100 higher concentrations[6] or for longer application periods under occlusion.[7,8]

It is well documented that reproducibility of TEWL is dependent on the body site.[18–20] Most previous studies on the influence of detergents were carried out on the volar forearms, mainly because of the high reproducibility of TEWL in this region. However, this location seemed inappropriate for our purposes, because forearm skin is not the major target of detergent-induced skin changes during daily dish-washing. We therefore decided to determine TEWL in the middle of the dorsal aspects of the hands. Prior to the study the mean TEWL in our group was higher than reported for the back,[10,21] and the forearm,[6,7,9,12] but lower than on vulvar skin[9] and the palms.[15,18] In pilot experiments in five different volunteers not included in this study, the reproducibility of TEWL on the dorsa of the hands was found to be sufficient ($CV < 20\%$, data not shown). Because TEWL at baseline was the same in EH and CH and relative TEWL values (i.e. TEWL on EH minus TEWL on CH after correction for baseline) were positive numbers in 16/18 volunteers, differences arising during the study can be attributed to different treatments. Furthermore, changes of TEWL in CH did not exceed 20% in all but two of our volunteers. This is not only additional proof of sufficient reproducibility, but also shows that hand protection with rubber gloves generally does not induce significant barrier alteration. In two subjects, increase of TEWL values of CH were substantial but still much lower than in the contralateral EH. Minor mechanical trauma, occlusive effect of the rubber gloves and exposure to warm ambient temperature are the most likely causes, although factors unrelated to the study cannot be ruled out.

Whether changes in the epidermal barrier caused by daily dish-washing are due to the irritant effect of the detergent itself, to additional irritating compounds present in the washing-up liquid (e.g. solvents such as urea) or to the effect of water (pH, ion strength etc.) cannot be completely answered by our results. The results of the control experiment, however, in which the three volunteers who had exhibited the most severe reactions to the washing-up liquid soaks were exposed to warm tap-water, without experiencing any significant reactions, does not favour a prominent role for water alone.

In agreement with previous studies,[7] we observed a decrease of TEWL and clinical symptoms after the second week of the study. This decrease is most likely due to adaptation ('hardening of the skin').

A high baseline TEWL has been reported to be a good predictor of susceptibility to irritants on the volar forearm.[8,22] In our volunteers, on the dorsum of EH a strong trend for correlation was found between TEWL at baseline and after 1 week ($P \leqslant 0{\cdot}05$; Spearman rank). TEWL may thus indeed be a good screening parameter for chemically vulnerable skin, even under everyday life conditions with many other factors than detergents having an impact on the epidermal barrier.

Six volunteers had elevated IgE, but only one gave a history of atopic eczema, and there were no clinical signs of disease at the beginning of the study. With regard to baseline TEWL, TEWL increase, relative TEWL and presence of objective clinical signs and subjective symptoms during the study, those with elevated IgE did not differ from the other volunteers. This supports previous findings, that persons with atopic symptoms such as rhinitis or asthma, without atopic dermatitis, are not at increased risk of developing changes of the epidermal barrier after detergent use.[22] Furthermore, IgE levels seem to be a poor predictor of skin reactivity. This observation should be substantiated in a larger sample.

In summary, our results demonstrate that regular exposure to low concentrations of detergents, as used for dish-washing, is capable of inducing objective and subjective skin lesions in a large proportion of the population. It is likely that many of the people thus affected are specifically predisposed to barrier defects; this predisposition may be predicted by the use of TEWL measurements. In everyday life, these changes probably do not become clinically apparent in most people, due to the use of protective measures.[1] The notion cannot be maintained, however, that detergent use *per se* is innocuous to the skin and does not play a relevant role in the causation of housewife's eczema. It is likely that many people who are exposed to detergents experience minor or moderate adverse reactions before adaptation takes place and the appropriate protective measures become part of the daily routine. This might be a simple explanation for the negative image of washing-up liquids in the public mind which may otherwise too easily be dismissed as prejudice.

Acknowledgment

This work was supported by the Arbeitsgemeinschaft für Hygiene und Umwelt, Vienna, Austria.

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British Journal of Dermatology (1992) 127, 138–146.

Cutaneous manifestations of the eosinophilia–myalgia syndrome

JUDITH R.OURSLER,* E.R.FARMER,* R.ROUBENOFF,† H.S.MOGAVERO‡ AND ROSEMARIE M.WATSON*

*Departments of * Dermatology and † Rheumatology, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.*
‡ Buffalo Medical Group, P.C., 85 High Street, Buffalo, NY, U.S.A.

Accepted for publication 9 March 1992

Summary We report the cutaneous manifestations of the eosinophilia–myalgia syndrome in 10 patients, with specific reference to their clinical course, histopathological features, and immunogenetic studies. Cutaneous manifestations could be classified into three groups: morphoea-like sclerosis, urticarial and papular lesions, and generalized sclerosis. Despite this polymorphic clinical presentation, the histopathological abnormalities in all cases were strikingly similar, and consisted of superficial and deep perivascular lymphocytic dermal infiltrates, mucin deposition, and fascial inflammation (often in the absence of sclerosis). Immunoperoxidase studies revealed increased numbers of factor XIIIa- and MAC 387-positive cells in the inflammatory infiltrate. Immunogenetic studies demonstrated that 77% (7/9) of patients possessed the HLA-DR3 or HLA-DR4 phenotypes. Mean follow-up of 24 months after discontinuation of L-tryptophan revealed the presence of persistent severe disabling disease in 30% of patients.

The eosinophilia–myalgia syndrome (EMS) is a recently described disease defined by (i) peripheral eosinophil count $>1{\cdot}0\times10^9$/l, (ii) severe myalgia interfering with daily activities and (iii) absence of infectious or neoplastic causes of eosinophilia or myalgia.[1] Other manifestations of EMS may include fever, arthralgia, dyspnoea, neuropathy, peripheral oedema and skin lesions. This syndrome has been linked with the ingestion of the amino acid L-tryptophan for insomnia, depression, premenstrual symptoms, and body building.[2] EMS is a disabling and often chronic disease that reached epidemic proportions after its initial report in November 1989. Over 1500 cases have been reported to the CDC, with 27 associated deaths.[3]

Although the aetiology of EMS remains unclear, there is growing evidence that the cause may be a contaminant, introduced during bacterial fermentation in the manufacture of L-tryptophan.[4,5] There is also some evidence for abnormal metabolism as a contributory factor.[6,7,8]

Various skin lesions have been described in case reports of EMS. We have studied the clinical and cutaneous histopathological features of 10 patients with EMS, and compared their histological features with eosinophilic fasciitis, morphoea, and scleroderma. We also report the spectrum of extracutaneous clinical manifestations, laboratory data, HLA phenotypes and long-term response to therapy in these 10 patients.

Methods

Ten patients with probable EMS seen at the Johns Hopkins Hospital between September 1989 and September 1990 were evaluated for the study. Seven patients fitted the strict CDC criteria for EMS. The remaining three patients (Nos 2, 4 and 8) had disabling myalgia and eosinophilia attributed to ingestion of L-tryptophan, but had absolute eosinophil counts of $<1{\cdot}0\times10^9$/l. They were accepted into our study, however, because they had significant eosinophilia (1·5, 4 and 6·8 × normal). Patients were examined by the authors, and information was obtained through medical-record review, and telephone conversations with patients and their primary physicians. Skin biopsy specimens were available retrospectively from all 10 patients, including fascial biopsies in seven. Two patients (Nos 1 and 7) underwent two biopsies separated by intervals of 3 and 2 months, respectively. The specimens were taken from various anatomical sites and an estimate of the duration of lesions at these sites was made. Histological studies were performed on each specimen

Correspondence: Dr Rosemarie M.Watson, Department of Dermatology, Johns Hopkins Hospital, Blalock 920, 600 N. Wolfe Street, Baltimore, MD 21205, U.S.A.

with haematoxylin and eosin, Giemsa, Leder, and colloidal iron stains performed according to the AFIP methods.[9] Immunoperoxidase stains (factor XIIIa and MAC 387) were performed with the reagents and protocol of Vector Laboratory's Vecta stain ABC kit.[10] The same procedure using normal rabbit serum was performed as a control. Immunoperoxidase staining of normal skin and fascia was also performed as a negative control. In addition, six biopsies with well characterized morphoea ($n=4$), scleroderma ($n=1$), and eosinophilic fasciitis ($n=1$) were reviewed for comparison, and these patients were questioned to ensure absence of L-tryptophan ingestion. All cases were reviewed by a single dermatopathologist (E.F.).

Antinuclear antibody testing was performed by indirect immunofluorescence using HEp-2 cells as a substrate and staining with fluorescein-conjugated antisera (Kallestad Laboratories Inc.). HLA-A, -B, and -C specificities were determined by a standard complement-dependent microcytotoxicity test, and serological typing for HLA-DR and -DQ antigens was performed using a double-fluorescence microcytotoxicity test.[11] HLA antisera were standardized against those of the 8th and 9th International Histocompatibility Testing Workshops. Controls consisted of 3761 (HLA-A, -B, and -C) and 1094 (HLA-DR, -DQ, -DRw 52, and DRw 53) local Caucasians. Statistical analysis of HLA phenotypes was performed using a two-tailed Fisher test. Case descriptions of Patients 1 and 9 have been previously reported.[12]

Case reports

The cutaneous manifestations in our patients could be categorized into three groups: morphoea-like sclerosis, urticarial and papular lesions, and generalized sclerosis. Three representative cases, one from each category of cutaneous disease, are described in further detail below, and illustrated in Fig. 1.

Patient 1

A 34-year-old Caucasian woman who had been taking 2–4 g of L-tryptophan daily since January 1988, was well until September 1988 when she noted oedema and paraesthesia in her legs. She then developed tender 'bruises' on her legs (Fig. 1a), which progressed to involve her arms, buttocks and lower trunk over the next year. Intense myalgia prompted her to leave her job as a waitress and stop daily weight training. Examination revealed multiple 1–5 cm plaques of hyperpigmented morphoea-like sclerosis on her thighs, calves and trunk. Her leucocyte count was $9{\cdot}8 \times 10^9$/l with an absolute eosinophil count of $1{\cdot}2 \times 10^9$/l. Skin biopsy revealed superficial and deep perivascular infiltration with lymphocytes, plasma cells and eosinophils, and a fasciitis. One month after discontinuing L-tryptophan, her cutaneous lesions had faded, but she continued to have disabling myalgia. The myalgia improved slowly on treatment with 10–20 mg of oral prednisone daily, but then worsened, and was accompanied by new skin

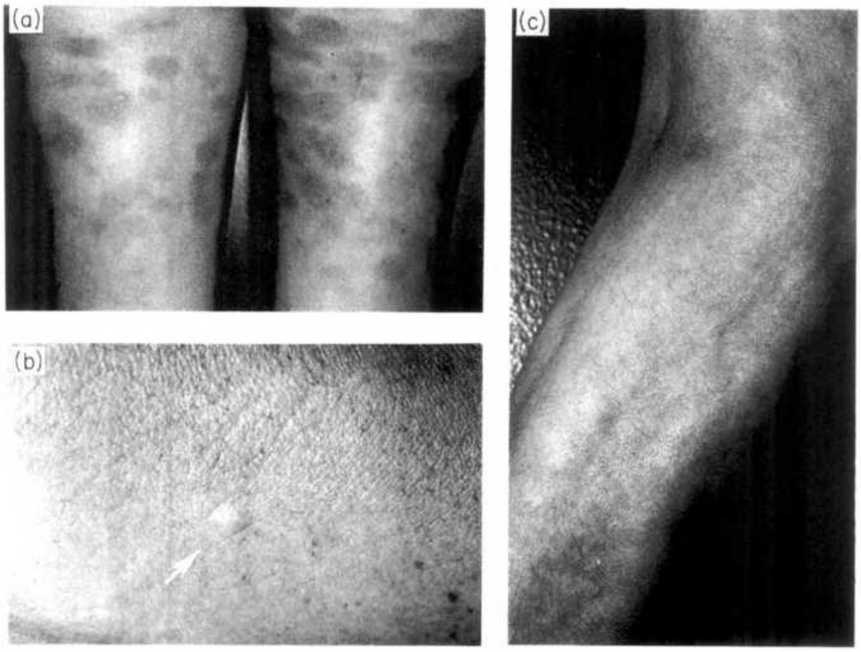

Figure 1. (a) Patient 1, morphoea-like sclerosis; (b) Patient 7, firm papule; (c) Patient 9, generalized sclerosis.

lesions, when the prednisone was decreased to 7·5 mg daily. Hydroxychloroquine (Plaquenil®) 200 mg b.d. was added from August 1989 to August 1990, with subsequent slow improvement in her skin lesions. By September 1990 her lesions had faded, but she still had significant myalgia. She no longer walked with a limp, but had occasional arthralgia in her knees on exercise and had only 4+/5 strength in her extremities. In January 1990 the hydroxychloroquine was replaced by chlorambucil 6 mg/day. She continued on 15 mg/day of prednisone. Her gradual improvement continued, with increased muscle strength and softening of all skin lesions, but hyperpigmentation persisted. Chlorambucil was discontinued because of significant leucopenia, and methotrexate 15 mg/week was commenced. She continued on the same steroid dose. Her disease flared, with recurrent fever, weakness, fatigue, paraesthesiae, dyspnoea, and new cutaneous lesions (described as 'waxy bruises'). Methotrexate was discontinued and prednisone was increased to 100 mg/day, with resolution of her dyspnoea, and some improvement of her other symptoms. Her current dose of prednisone, 20 months after stopping L-tryptophan, is 20 mg on alternate days. However, she is still unable to work and remains in bed much of the time due to fatigue and weakness. Cutaneous sclerosis and hyperpigmentation are still present, but improved.

Patient 7

A 38-year-old Caucasian woman had been taking 1500 mg of L-tryptophan daily for 8 months as a hypnotic. She developed fatigue, myalgia, waxy papules and urticaria in October 1989. Her leucocyte count was $19{\cdot}1\times10^9$/l with an absolute eosinophil count of $8{\cdot}35\times10^9$/l. L-tryptophan was discontinued and she was treated with oral prednisone 50 mg/day, with improvement in all her symptoms. She developed new firm papules on her arms in January 1990 after steroid therapy was discontinued, and her urticaria recurred in February. Examination revealed multiple 2–5 mm flesh-coloured papules over both forearms (Fig. 1b). A biopsy showed mucin deposition in the reticular dermis, subcutis and fascia, and a mild perivascular lymphocytic infiltrate. In September 1990 steroids were resumed at a dose of 15 mg daily, and hydroxychloroquine 200 mg b.d. was added, with subsequent improvement. All her skin lesions resolved, leaving post-inflammatory hyperpigmentation. Her daily activities were no longer restricted, and she had no difficulty climbing stairs, but she did complain of excessive fatigue after attending martial arts classes.

Patient 9

A 48-year-old Caucasian woman had been taking 750–1500 mg of L-tryptophan daily as a hypnotic for 14 months. In July 1989, she noted difficulty keeping her mouth open for dental procedures. She then developed generalized sclerosis (Fig. 1c), myalgia, dyspnoea and fever. A diagnosis of EMS was made in November 1989. Initial examination disclosed waxy induration of the skin on the extremities, face and trunk. Her leucocyte count was $16{\cdot}1\times10^9$/l with an absolute eosinophil count of $8{\cdot}5\times10^9$/l. A skin biopsy revealed telangiectasia and oedema of the reticular dermis with a diffuse interstitial infiltrate of lymphocytes, mononuclear cells and eosinophils, and a fasciitis. L-tryptophan was discontinued, and steroids commenced. She subsequently developed pleurisy and neuropathy, but these subsided gradually on steroid therapy.

In December 1991 she was taking only 2·5 mg prednisone on alternate days. She was able to swim up to a quarter of a mile, and had no myalgia or dyspnoea. However, she has severe fatigue, pruritus, and persistent sclerosis of forearms and legs. Her disability has prevented her from returning to her previous occupation as a teacher. She has recently begun a part-time sedentary occupation.

Results

Clinical features

The patients' demographic profiles and their clinical features are summarized in Table 1. All 10 patients studied were women with a mean age of 49 years (range 32–72). They developed symptoms 8–36 months (mean 16·6 months) after tryptophan therapy was commenced. The daily doses of tryptophan ranged from 0·5 to 3·0 g (mean 1·7 g), and the mean cumulative dose was 1021·5 g (range 248–3240 g). Consistent with the national epidemiology reported in EMS, 90% of our patients were Caucasian, 100% were women, and 70% fell within a 35–54-year age group (range 32–72).

The past medical histories of our patients are summarized in Table 2. Five patients were taking a variety of additional medications when their symptoms first occurred. Two patients had prior histories suggestive of underlying connective tissue disease. Patient 1 had a single episode of transient arthralgia with a positive rheumatoid factor 3 years prior to her EMS. Patient 8 had a biopsy-confirmed diagnosis of Sjögren's syndrome a few months prior to the diagnosis of EMS.

Table 1. Patient characteristics and clinical features of 10 patients with the eosinophilia–myalgia syndrome*

				Tryptophan		Laboratory data		
Patient	Age/Sex/Race*	Lesion type	Lesion site	Dose (g)†	Duration (months)	EOS‡ (×10⁹/l)	ANA	ESR
1	37/F/W	Morphoea-like	Legs, arms, trunk	3240	36	1 2	1.640	12
2	54/F/H	Morphoea-like	Legs, arms, trunk	NA	12	0·35	Neg.	13
3	45/F/W	Morphoea-like	Face, legs, arms, trunk	1620	18	3·9	Neg.	20
4	37/F/W	Morphoea-like	Face, legs, arms	600	30	4·5	1:160	50
5	32/F/W	Urticaria, papules	Legs, arms	248	11	0·89	Neg.	40
6	57/F/W	Papules	Legs, arms	NA	3	2·18	Neg.	5
7	38/F/W	Urticaria, papules	Arms	360	8	8·35	Neg.	8
8	68/F/W	Sclerotic, hypopigmented	Legs, trunk	1080	36	0·48	1:640	8
9	48/F/W	Sclerotic	Legs, arms, trunk	574	17	8·5	Neg.	9
10	72/F/W	Sclerotic	Legs, arms	450	6	6·3	1:640	30

*W, White; H, Hispanic. †Dose, total estimated cumulative dose. ‡EOS × 10^9/l, highest recorded absolute eosinophil count. NA, not available.

Cutaneous manifestations

Cutaneous disease was present in all cases, and included findings which could be classified into three general categories: morphoea-like sclerosis (Patients 1–4), urticarial and papular lesions (Patients 5–7), and generalized sclerosis (Patients 8–10). These occurred as specific entities and not as a progression of one type of lesion into another within one patient. For example, those patients who had generalized sclerosis did not begin with morphoea-like lesions and develop widespread disease, but developed their initial cutaneous signs as a generalized eruption. Most cutaneous lesions were concentrated acrally, although the trunk and face were also involved in those patients with generalized sclerosis. Alopecia (independent of steroid therapy) was noted in three cases, but was transient and non-scarring in all instances. Patients with generalized sclerosis were clinically indistinguishable from patients suffering from eosinophilic fasciitis. Differentiation from systemic sclerosis was on clinical grounds, and aided by the absence of sclerodactyly, telangiectasia, and Raynaud's phenomenon.

Table 2. Medical history of patients with EMS

Patient	
1	Hysterectomy; transient arthralgia; insomnia; history of substance abuse. No medication
2	Depression; benign thyroid nodule; recurrent giardiasis. Medications include fluoxetine (Prozac®), maprotiline hydrochloride (Ludiomil®), levothyroxine sodium (Synthroid®) spironolactone
3	Photosensitivity; multiple laminectomies; insomnia; syndrome X (small vessel coronary artery spasm); hypothyroidism; hysterectomy with bilateral salpingo-oophorectomy. Medications include verapamil, oestrogen, isosorbide dinitrate (Isordil®), levothyroxine sodium (Synthroid®), ranitidine, acetaminophen/oxycodone hydrochloride (Percocet®), nifedipine
4	Bulimia. No medications
5	No significant medical history. No medications
6	No significant medical history. Medications include medroxyprogesterone acetate (Provera®), oestrogen, pyridoxine
7	Basal cell carcinoma. No medictaion
8	Sjögren's syndrome; thyroidectomy; depression. Medications include levothyroxine sodium (Synthroid®)
9	Hysterectomy; vitiligo; ankle fusion; insomnia. Medications include medroxyprogesterone, oestrogen, calcium, vitamin D, vitamin C
10	Depression; insomnia Medications include meprobamate

Extracutaneous manifestations

All patients suffered from myalgia and fatigue. Other symptoms included oedema (70%), neuritis (40%), arthralgia (20%), and fever (20%). Pulmonary dysfunction, the most common cause of EMS-related death was seen in 40% of our patients and ranged from mild dyspnoea to life-threatening ventilatory defects.

Treatment and course

All patients stopped taking L-tryptophan, and seven were treated with prednisone (doses ranging from 10 to 100 mg daily). Three of these patients responded promptly, but four continued to have difficulties. Many patients' symptoms worsened when their dose of prednisone was reduced to below 20 mg/day. Additional therapies such as hydroxychloroquine sulphate, chlorambucil, cyclosporin, and azathioprine were used in severe cases, with variable results. Discontinuation of chlorambucil due to leucopenia resulted in a severe flare in Patient 1. Intravenous gammaglobulin (IVIG) was used in two patients and plasmapheresis in one. After brief courses of IVIG, Patients 3 and 10 had increases in

Table 3. Follow-up of patients with the eosinophilia-myalgia syndrome

Patient	Number of months off tryptophan	Treatment and response (0–3)*	Current status
1	24	Prednisone, maximum 100 mg daily (2) Hydroxychloroquine 200 mg b.d. (1) Methotrexate 15 mg weekly (0) Chlorambucil 6 mg daily (3)†	Unable to work Prednisone 20 mg alt. days Fatigue, myalgia Modest improvement in sclerosis and hyperpigmentation
2	24	Not available	Not available
3	26	Prednisone 70 mg daily (1) IV prednisone pulse 100 mg daily × 3 days (1) Intravenous immunoglobulin 0·4 g/kg × 5 days (0) Azathioprine 250 mg daily (0)	Prednisone 20 mg daily Wheelchair-bound Persistent severe myalgia, stiffness, dyspnoea and neuropathy
4	25	No treatment	Resolution of morphoea Persistent pigmentation Returned to work
5	20	Prednisone 60 mg daily (1) Amitriptyline 25 mg t.d.s. (2)	Off prednisone Sclerosis improved Dyspnoea improved Returned to work
6	18	No treatment	Returned to work Improvement in leg sclerosis Pigmentation and leg cramps persist
7	26	Prednisone 50 mg tapering (3) Hydroxychloroquine 200 mg b.d. (2)	Returned to work Off prednisone and hydroxychloroquine Sclerosis resolved Pigmentation persists Mild fatigue and myalgia
8	30	Prednisone 50 mg daily (2) Plasmapheresis (1) Cyclosporin A 100 mg b.d. (0) Azathioprine 150 mg daily (2) Quinine sulphate 260 mg q.h.s. (2)	Retired since onset of illness Prednisone 20 mg alt. days Azathioprine 150 mg daily Walks with cane Resolution of dysphagia Neuropathy improved, but symptomatic Sclerosis, legs only Hyperpigmentation persists
9	25	Prednisone 60 mg daily (2) Hydroxychloroquine 200 mg daily Doxepin HCl 50 mg daily (3, pruritus only) Cyclobenzaprine HCl 10 mg t.i.d. (2, cramps)	Return to part-time work only Prednisone 2·5 mg daily, other medications unchanged. Modest overall improvement Severe fatigue, leg cramps and pruritus persist
10	24	Prednisone 50 mg daily (2) Intravenous immunoglobulin (0) (0·4 g/kg/day × 5 days) Azathioprine 100 mg daily Azathioprine 150 mg daily (3)	Marked gradual improvement Off prednisone Azathioprine 100 mg daily Walks unaided Neuropathy and myopathy resolved Leg sclerosis persists

* 0, no response; 1, minimal; 2, modest; 3, significant.

† Chlorambucil was discontinued because of leucopenia after 8 months, with prompt flare of symptoms.

their forced vital capacity from 0·7 to 1·04 l, and from 0·5 to 0·7 l, respectively. Patient 8, however, had only minimal improvement in her neuropathy manifested as decreased numbness and weakness after an eight-cycle course of plasmapheresis. Severe pruritus and cramps were controlled by doxepin hydrochloride and cyclobenzaprine hydrochloride, respectively, in Patient 9. Table 3 summarizes the follow-up data and treatments used in our patients.

Histology

The histological findings of our patients and comparison patients are summarized in Table 4. The most characteristic findings were perivascular lymphocytic infiltrates, fibrosis, fasciitis (see Fig. 2), and mucin deposition without sclerosis. This is in contrast with comparison patients with morphoea, scleroderma and eosinophilic fasciitis, in whom no mucin was seen, and sclerosis was often a prominent feature. Lymphocytic infiltrates often extended from the dermis through the subcutis, and the infiltrate sometimes included eosinophils. Special stains were remarkable for a large preponderance of factor XIIIa- and MAC 387-positive cells within the inflammatory infiltrates of the EMS patients. These were only sporadically seen in the comparison patients. One patient (6) had a distinctive perineural infiltrate, but did not have any clinical evidence of neuropathy.

HLA antigens

The HLA phenotypes available for our patients are summarized in Table 5. Seven out of 9 (77%) patients had either HLA-DR3 or HLA-DR4 phenotypes. HLA-DR4 was present in 44% (4/9) and HLA-DR3 in 33% (3/9) patients. No significant differences were noted when the frequencies were compared with normal controls. An increased frequency of the HLA-A1, -B8, -DR3 phenotypes (33%, 3/9) ($P = 0{\cdot}02$) was noted. The probability that these phenotypes are represented on the same haplotypes was calculated as 95% (Patient 6), 95% (Patient 7), and 86% (Patient 10), respectively.[13]

Discussion

The cutaneous lesions of our patients may be classified into three general categories: morphoea-like sclerosis,

Table 4. Histopathology

		Inflammation					Mucin				
Patient	Months*	D	S	F	eos.	Thick fascia	D	S	F	FXIIa	MAC 387
EMS patients											
1	11	+	+	NA	+	NA	NA	NA	NA	NA	NA
	14	+	+	+	−	+	−	−	+	+	+
2	0	+	+	NA	+	NA	+	NA	NA	NA	NA
3	4	+	+	+	−	+	−	+	+	+	+
4	3	+	−	NA	−	NA	+	+	NA	+	+
5	3	+	NA	NA	+	NA	+	NA	NA	+	+
6	1	+	−	+	−	+	+	−	−	+	+
7	4	+	+	−	−	−	+	+	+	+	−
	6	+	−	−	−	−	−	+	−	NA	NA
8	5	+	−	+	−	−	−	+	−	+	−
9	11	−	NA	NA	−	−	+	+	+	+	+
10	4	+	−	+	−	−	−	−	−	−	−
Comparison patients											
1	EF	+	−	+	−	+	−	−	−	−	−
2	M	+	NA	NA	−	NA	−	NA	NA	Scl	Scl
3	M	+	−	NA	−	NA	+	−	NA	Scl	Inflam.
4	M	+	NA	NA	−	NA	+	NA	NA	Scl	Scl
5	M	+	−	NA	−	NA	+	−	NA	Scl and inflam.	Inflam.
6	S	+	NA	NA	−	NA	+	NA	NA	+	+

* Number of months after patient's first symptoms that biopsy was taken; eos, eosinophils; D, dermal; S, subcutaneous; F, fascial; NA, not available; EF, eosinophilic fasciitis; M, morphoea; S, scleroderma; Scl, + in sclerotic areas; Inflam, + in inflammation.

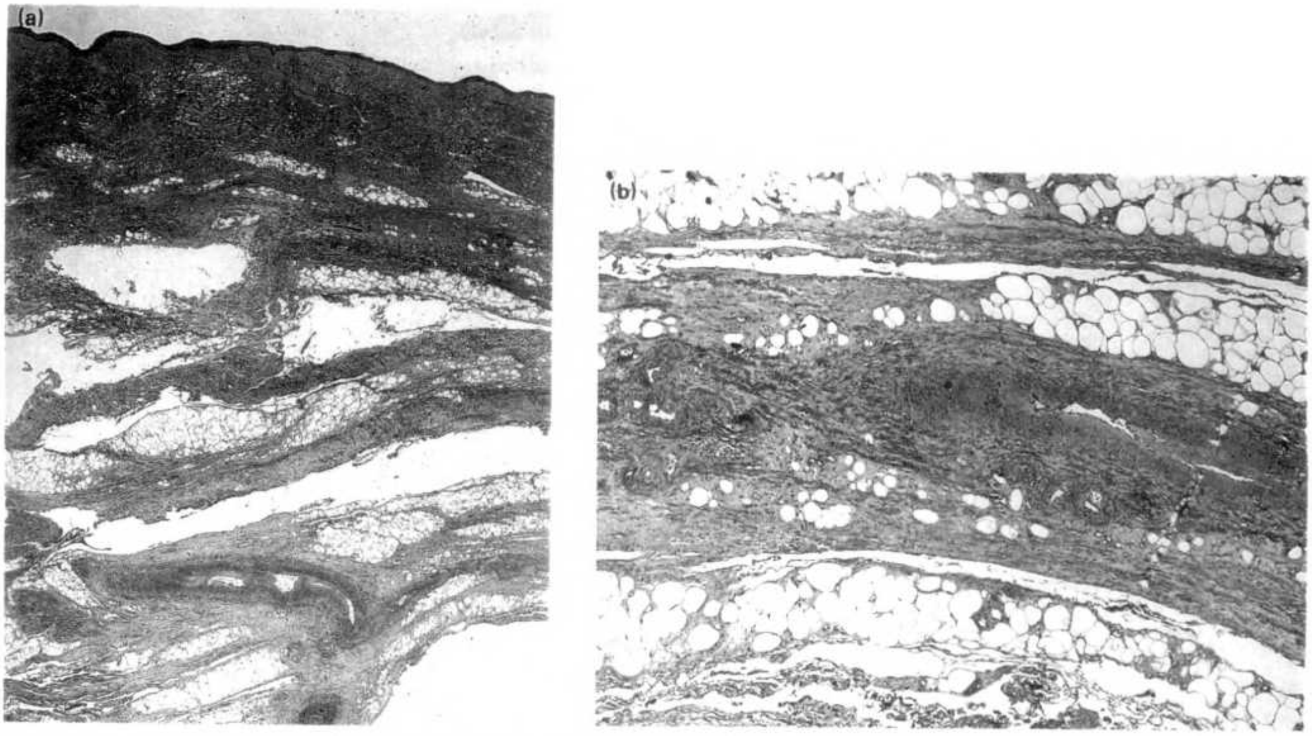

Figure 2. (a) Low-power photomicrograph demonstrating fascial thickening and fibrosis replacing the subcutis in eosinophilia–myalgia syndrome (haematoxylin and eosin × 7). (b) High-power photomicrograph demonstrating fibrosis replacing the subcutis in eosinophilia–myalgia syndrome (haematoxylin and eosin × 45).

Table 5. HLA phenotypes in eosinophilia–myalgia syndrome patients

Patients	HLA phenotypes						
1	A2.3	B60.44	Bw4.w6	C3.4	DR4.7	DQ2.3	DRw53
3	A1.24	B62.60	Bw6	C3	DR4.6	DQ1.3	DRw52.53
4	A2.28	B51	Bw4	C4	DR4.11	DQ3.7	DRw52.53
5	A2.26	B27.38	Bw4	C2	DR4.13	DQ3.1	DRw53.53
6	A1.26	B8.27	Bw4.w6	Cw4	DR3.8	DQ2.4	DRw52
7	A1.26	B8.7	Bw6	C7	DR2.3	DQ1.2	DRw52
8	A2	B27.18	Bw4.w6	C2	DR1.11	DQ1.7	DRw52
9	A1.3	B7	Bw6	Cw7	DR2.8	DQ1.4	
10	A1.11	B8.60	Bw6	C3.7	DR3.6	DQ1.2	DRw2

papular and urticarial lesions, and generalized sclerosis. All three categories of disease, as well as alopecia, have been independently reported in case reports of EMS.[14–17] Other skin lesions, which have been associated with EMS in case reports are erythroderma, morbilliform eruptions, oral ulcers, dermatographism and livedo reticularis. The skin lesions seen in our patients were thought to be related to EMS because of the timing of their development, the absence of any previous dermatological history, and also because skin lesions often improved with the patient's other symptoms and flared when other symptoms worsened (as in Patient 1). An explanation for lower eosinophil counts ($< 1{\cdot}0 \times 10^9$/l) in three of our patients may be that they were obtained 1, 1, and 6 months, respectively, after the patients had discontinued L-tryptophan.

Five of the seven patients (Nos 1, 3, 8, 9, 10) who had sclerotic changes (both localized and generalized) developed more severe systemic disease and had a worse long-term outcome than those patients with urticaria and papular lesions. No other factors, including dosage of L-tryptophan, HLA phenotype, eosinophil count, ANA, and ESR could be correlated with either prognosis or severity of systemic disease. Three of the five patients with severe disease and poor prognosis were found to have a history suggestive of a predisposition to autoimmune disease. This included: arthralgia, Sjögren's syndrome, hypothyroidism, and vitiligo. No other predisposing factors for type of skin lesion could be identified.

The most consistently effective therapy in our patients was discontinuation of L-tryptophan. Systemic steroids and antimalarial agents seemed to produce modest improvement. Some response to intravenous immunoglobulin (IVIG) in those patients with neuropathy was seen, and is consistent with the improvement in many types of neuropathies seen with IVIG therapy.[18] As already mentioned, Patient 1 had a flare of her disease when chlorambucil was discontinued. Some reviews have reported improvement with D-penicillamine, colchicine, methotrexate, and isotretinoin.[19] Despite aggressive therapeutic regimens, 55% (5/9) of our patients had disabling disease 1 year after discontinuation of L-tryptophan and 33% (3/9) at 2 years (Table 3). Of the six patients who had improved at 2 years, all had significant manifestations of one or more of the following: sclerosis (4/6), pigmentary changes (4/6), leg cramps (2/6), neuropathy (1/6) and myalgia (1/6).

Although biopsy specimens were obtained at varying intervals after the onset of skin lesions, they were all strikingly similar. All but one biopsy showed a perivascular lymphocytic infiltrate throughout the dermis. This infiltrate was also seen in subcutaneous septa in most specimens, and often in the fascia. Although the infiltrate was predominantly composed of lymphocytes, eosinophils, plasma cells, and histiocytic cells were occasionally present. Increased numbers of mast cells were seen in three patients. These results are similar to the study of Silver *et al.*[16] in which the infiltrate consisted mainly of lymphocytes, but eosinophils and plasma cells were sometimes present, and two of nine patients had increased numbers of mast cells. Factor XIIIa- and MAC 387-positive cells were almost universally increased within the inflammatory infiltrate of EMS, while they were only variably seen in the comparison diseases, and then often associated with sclerosis rather than inflammation. MAC 387 recognizes an antigen expressed by human myelomonocytic cells, and inflamed epidermis may stain with the antibody.[20] Factor XIIIa labels bone marrow-derived dermal dendritic cells that are distinct from Langerhans cells, and probably represent a specific immunological cell population found in increased numbers in inflamed skin.[21] These cells are capable of macrophage effector functions and antigen-presenting cell functions.[21] The finding of increased numbers of MAC 387 and factor XIIIa-positive cells in our patients is of interest. These cells are also increased in a wide variety of skin diseases and, thus, are not specific for eosinophilia–myalgia syndrome. The immune capabilities of these cells suggests a potential role in the pathophysiology of these disorders.

Despite the clinical similarity of many of our patients' lesions to morphoea and scleroderma, histologically they were distinct, as dermal sclerosis was not a component. However, EMS patients did show mucin deposition in the dermis, subcutis and fascia, and this finding has been noted in other studies of EMS.[22,23] Biopsy specimens from two patients included skeletal muscle. Both showed some degree of muscle degeneration, and one had active myositis.

HLA studies in our patients revealed that 74% (7/9) possessed either the HLA-DR3 or -DR4 phenotypes (HLA-DR3, 33%, 3/9; HLA-DR4, 44%, 4/9). In addition, an increase in the HLA-A1, -B8, -DR3 phenotypes was seen (33%, 3/9, $P = 0{\cdot}02$). These findings are of interest in view of the association of these phenotypes with autoimmune disease, notably, lupus erythematosus, C4 isotype deficiences (HLA-A1, B8, DR3)[24,25,26] and rheumatoid arthritis (HLA-DR4).[27,28] HLA-DR4 was also found in 55% of EMS patients studied by Kaufman *et al.*,[22] (controls 37%, $P =$ NS). Varga *et al.*,[29] noted a statistically insignificant increase in HLA-B7 phenotypes in their patients (50% vs. 19% of controls). HLA-B7, present in 22% (2/9) of our patients was comparable with controls. Our HLA studies and those of Kaufman *et al.*,[22] and Varga *et al.*,[29] suggest EMS patients are a heterogenous group. Studies of larger numbers of patients will be required to determine if the increase in HLA-DR4 and/or -A1, -B8, -DR3 phenotypes seen in our patients is statistically significant, suggesting a hereditary predisposition in the aetiology of this syndrome.

Acknowledgments

The authors thank Ms Marcella Sutherland for technical assistance with immunoperoxidase staining, Ms Susan Weinstein for technical assistance with histopathology, Dr Alfred M.Stein for obtaining HLA phenotypes of Patients 4 and 5, and Dr Wilma Bias for providing HLA phenotypes on the other patients.

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British Journal of Dermatology (1992) 127, 147–154.

A portable pulsed electromagnetic field (PEMF) device to enhance healing of recalcitrant venous ulcers: a double-blind, placebo-controlled clinical trial

M.J.STILLER, GRACE H.PAK, J.L.SHUPACK, S.THALER,* CLARE KENNY AND LORRIE JONDREAU

The Ronald O.Perelman Department of Dermatology, New York University Medical Center, Dermatopharmacology Section and Leg Ulcer Clinic, 550 First Avenue, New York, NY 10016, U.S.A.
**Geomed. Inc., 603 Chestnut St, Garden City, NY 11530, U.S.A.*

Accepted for publication 8 April 1992

Summary

A prospective, randomized, double-blind, placebo-controlled multicentre study assessed the clinical efficacy and safety of pulsed electromagnetic limb ulcer therapy (PELUT) in the healing of recalcitrant, predominantly venous leg ulcers. The portable device was used at home for 3 h daily during this 8-week clinical trial as an adjunct to a wound dressing. Wound surface area, ulcer depth and pain intensity were assessed at weeks 0, 4 and 8. At week 8 the active group had a 47·7% decrease in wound surface area vs. a 42·3% increase for placebo ($P<0·0002$). Investigators' global evaluations indicated that 50% of the ulcers in the active group healed or markedly improved vs. 0% in the placebo group, and 0% of the active group worsened vs. 54% of the placebo group ($P<0·001$). Significant decreases in wound depth ($P<0·04$) and pain intensity ($P<0·04$) favouring the active group were seen. Patients whose ulcers improved significantly after 8 weeks were permitted to continue double-blind therapy for an additional 4 weeks. Eleven active and one placebo patient continued therapy until week 12, with the active treatment group continuing to show improvement. There were no reports of adverse events attributable to this device. We conclude that the PELUT device is a safe and effective adjunct to non-surgical therapy for recalcitrant venous leg ulcers.

In the U.S.A. it is estimated that leg ulcers, 90% of which are due to venous stasis, affect 0·6% of men and 2·1% of women between 60 and 69 years of age.[1] The prevalence of leg ulcers increases to 5% by the age of 90.[2] An epidemiological study in the U.K. involving nearly 1500 patients with chronic leg ulcers estimated an incidence of 1·48/1000 population.[3] The duration of ulcers reported in such studies ranges from a few months to decades with a mean duration usually greater than 10 years.[1]

The paucity of alternative non-surgical therapies for ulcers that do not respond to conventional treatment provided the impetus for this study. Our interest in the application of low-frequency, low-energy pulsed electromagnetic fields (PEMFs) to promote wound healing stems from the following: (i) successful use of PEMFs in healing more than 100,000 non-union fractures,[4,5,6] (ii) anecdotal reports of acclerated closure of chronic skin wounds overlying fractures being treated with PEMFs,[7,8] (iii) growing acceptance of experimental data demonstrating the biological effects of low-energy electromagnetic fields,[4,9,10] and (iv) historical perspective on electrical stimulation of skin to enhance wound healing.

The use of electricity as a therapy for surface wounds can be traced back more than 300 years to the use of charged gold leaf to prevent smallpox scars.[11] Interest in electrical therapy was renewed in the mid-twentieth century with the discovery of electrical properties of living systems and the use of direct current (DC) stimulation to alter surface wound healing.[12] In 1983, Foulds and Barker demonstrated the presence of a 'battery' in human skin, i.e. an electrical potential difference across the epidermis, which becomes accentuated at the periphery of the wound following injury. This increased potential difference is thought to play a role in wound repair.[13] Human clinical trials and animal experiments have shown that electrical stimulation of skin accelerates wound healing, presumably by

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Presented in part at the Annual Meeting of the Society for Investigative Dermatology, Seattle WA, May 1 1991, and published as an abstract (*Journal of Investigative Dermatology* 1991; 96: 570, and *Clin Research* 1991; 39: 509A).

augmenting the endogenous current induced by injury.[12,14,15,16] However, DC stimulation used in these studies has the disadvantage of requiring electrode placement directly in or near the wound.

PEMFs have an inherent advantage over DC systems in that the electromagnetic signal penetrates the dressing and the tissue involved. In short, pulsed magnetic fields interact with electrically conductive elements in tissue, resulting in induced currents. The basic mechanisms underlying the clinical effects of PEMFs are not clear. However, it has been suggested that PEMFs, by altering or augmenting pre-existing endogenous electrical fields, may trigger specific, measurable cellular responses such as DNA synthesis, transcription and protein synthesis.[9] Such cellular responses appear to occur within a 'window' of PEMF parameters (frequency, amplitude, timing and length of exposure).[4]

It has been reported that PEMF stimulation decreases the doubling time of fibroblasts and endothelial cells and induces differentiation of skin fibroblasts in culture.[17–19] Increased collagen synthesis, angiogenesis, and bacteriostasis are some mechanisms by which PEMFs may contribute to wound healing.[17,18] *In vivo*, electrical stimulation has been shown to increase tensile strength of surgical wounds.[20] There are also reported effects of electromagnetic fields on hormonal regulation, membrane transport, enzymatic activity, and receptor expression.[21,22]

To date, one double-blind, placebo-controlled study in the orthopaedic literature has shown that PEMF stimulation enhances the healing of cutaneous ulcers.[23] Our clinical trial differs from the former study by the innovative features of the PEMF device including its light weight and portability, qualitative and quantitative parameters of the electromagnetic stimulus, as well as stringent protocol design. We report the results of a double-blind, placebo-controlled clinical trial to assess the efficacy and safety of pulsed electromagnetic limb ulcer therapy (PELUT) as an adjunct to non-surgical management of recalcitrant venous leg ulcers.

Methods

Study design

This study was designed as a prospective, multicentre, double-blind randomized trial, to evaluate the efficacy and safety of the PELUT device as an adjunctive treatment for recalcitrant venous stasis ulcers. The following eight centres participated: UMDNJ–Robert Wood Johnson Medical School, Department of Dermatology (New Brunswick, NJ), Vascular Skin Ulcer Institute (Chicago, IL), Lebanon Vascular Surgical Associates (Lebanon, PA), Pennsylvania College of Podiatric Medicine, Department of Medicine (Philadelphia, PA), VA Medical Center, Department of Podiatry (Lebanon, PA), Albert Einstein College of Medicine, Department of Vascular Surgery (Bronx, NY), NYU Medical Center, Department of Dermatology (New York, NY). Protocol-eligible patients were randomized according to a computer-generated code based on their order of admittance to the study. Each patient was assigned either an active or an indistinguishable placebo device according to the code devised by the study sponsor, Geomed, Inc.

Study population

Thirty-one patients, each with one study ulcer, were enrolled. Eighteen patients were randomized to the active treatment group and 13 patients to the placebo group. There were no statistically significant demographic differences between the placebo and active groups, as shown in Table 1.

A protocol-eligible wound consisted of a predominantly venous, full-thickness leg ulcer with maximum linear dimension (diameter) no greater than 7·0 cm, that had demonstrated unsatisfactory healing in response to non-surgical treatment for at least 4 weeks prior to enrollment in the study. The protocol also required ulcer stability, which was defined as 15% or less change in diameter and no more than 15% change in percentage of healthy granulation tissue during the 2-week period prior to the start of PELUT therapy.

Diagnosis of venous stasis ulcers was made within 1 month prior to the week 0 visit. Pertinent physical findings included oedema and varicosities and secondary signs of venous insufficiency such as stasis dermatitis, dependent rubor, hyperpigmentation and lipodermatosclerosis. Arterial disease was excluded by the presence

Table 1. Demographic data

	Active ($n=18$)	Placebo ($n=13$)
Mean age (years)	63·3	63·8
Age range (years)	41–87	39–76
Number of men	9	8
Number of women	9	5
Mean ulcer duration* (weeks)	38·9±5·2	46·8±11·3
Mean ulcer area (cm^2)	7·25±1·02	7·66±1·62

*Each subject had one designated study ulcer of strong pedal pulses and adequate capillary refill.[24] In addition, patients with a history of claudication, ischaemic heart disease, or cerebrovascular disease were excluded from the study. When necessary, Doppler ultrasound, photoplethysmography, impedance plethysmography, light reflection rheography, or arteriography were obtained to establish the diagnosis of venous insufficiency and to rule out arterial disease. Decubitus ulcers or ulcers primarily due to diabetes, vasculitis, neuropathy, infection or acute ischaemia were excluded from the study. Exclusion criteria also included patients with current active deep-vein thrombosis or thrombophlebitis, patients with pacemakers and patients suffering from alcoholism, nutritional deficiency, anaemia, congestive heart failure, hepatic or renal failure, uncontrolled diabetes, malignancy, or immunosuppression.

Protocol

At week 0 patients were instructed to apply the PELUT device over the wound dressing covering their ulcer for 3 h every day. This was done for 8 weeks or until the ulcer healed, if prior to 8 weeks. Patients were allowed to continue therapy to 12 weeks at the discretion of the investigator if the ulcer showed a favourable response at 8 weeks. This decision was made without breaking the patient's device assignment code. To monitor compliance, patients kept a diary listing start and stop time of each treatment application, which was reviewed by the investigator at the follow-up visits. The total hours of PELUT use was also calculated from the visual chronometer display on each device. To ensure proper functioning of the PELUT devices, each was inspected by the manufacturer prior to initiation of therapy and at the conclusion of treatment.

Table 2. Ancillary treatment

Treatment*	Active ($n=18$)	Placebo ($n=13$)
Duoderm®†	0	1
Gentamicin ointment + Duoderm®†	3	2
Mupirocin ointment + Vigilon®	2	1
Mupirocin ointment + non-adherent gauze	8	6
Elase® ointment + gauze	3	2
Unna's boot‡	2	1

* All regimens included an elastic compression wrap (Ace Bandage®) and leg elevation in addition to topical therapy

† Duoderm® hydroactive dressing is an occlusive, oxygen-impermeable sheet of hydrophilic particles encased in an inert hydrophobic polymer matrix placed on an adhesive plastic backing.[25]

‡ Unna's boot is a moist paste bandage impregnated with zinc oxide, calamine lotion and glycerin, wrapped snugly around the leg in direct contact with the ulcer, worn continuously and changed weekly. It provides topical wound therapy as well as compression. The boot produces a pressure gradient between ankle and calf, as do support stockings, yielding similar therapeutic efficacy in venous stasis ulcers.[26]

In addition to daily treatment with the PELUT device, all patients received ancillary topical treatment. The PELUT system was designed as an adjunctive therapy, so the topical treatment at each of the study centres was chosen by the investigator at each centre. Wound dressings used are summarized in Table 2. All regimens included an elastic compression wrap at all times (3-inch Ace Bandage®), which exerted approximately 20 mmHg pressure at ankle level. Leg elevation for a minimum of 3 h daily was also included in all ancillary treatment regimens. Patients who were unable to adhere to the requirement for leg elevation or whose occupation and/or life-style required prolonged standing were excluded from the protocol. The patients were seen in clinic at 2-week intervals to monitor safety and compliance. Efficacy parameters were assessed at weeks 0, 4, 8 (and 12), at which time photographs of the study ulcers were taken. The protocol was approved by the Institutional Review Boards of all participating institutions prior to initiation.

PELUT device

The PELUT system consisted of a control unit, containing a signal generator and a rechargeable 9-V battery, attached by an expandable cable to the electromagnetic transducer which was strapped over the wound dressing with an elasticized Velcro strap. The control unit and the transducer combined weighed 550 g (Figs 1 and 2). The transducer was an air coil employing magnetic focusing elements to provide improved efficiency (about 35%) and one-sided output. The PELUT system, which is designed and manufactured by Geomed Inc. delivers a low-energy PEMF that induces a low level, non-thermal electrical field of approximately 0·06 mV/cm. The signal is a 3-part pulse (+, −, +) of 3·5 ms total width and a duty cycle of 25%. The amplitude is bidirectional with delta B of approximately 22 Gauss. This bidirectional, quasi-symmetrical magnetic stimulus, in contrast to more frequently used unidirectional signals, minimizes energy consumption and patient exposure to peak magnetic fields. Parameters of the PELUT stimulus are derived from the PEMFs which have been used effectively to treat non-union fractures. Approximately 50% of these frac-

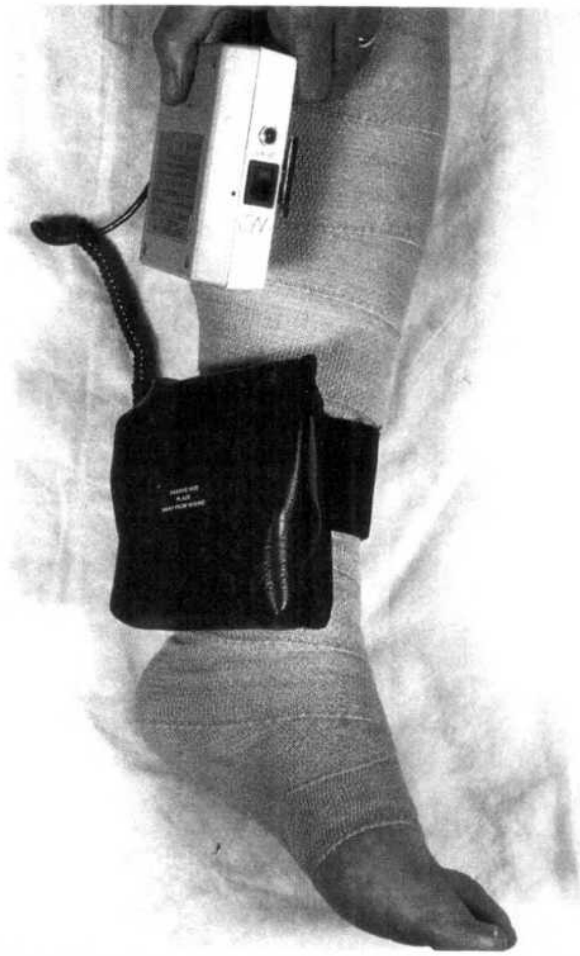

Figure 1. Pulsed electromagnetic limb ulcer therapy (PELUT) device. The electromagnetic transducer is placed over the wound dressing with an elasticized Velcro strap. The transducer is attached by an expandable cable to the control unit (signal generator and a rechargeable battery) which can be worn over a belt.

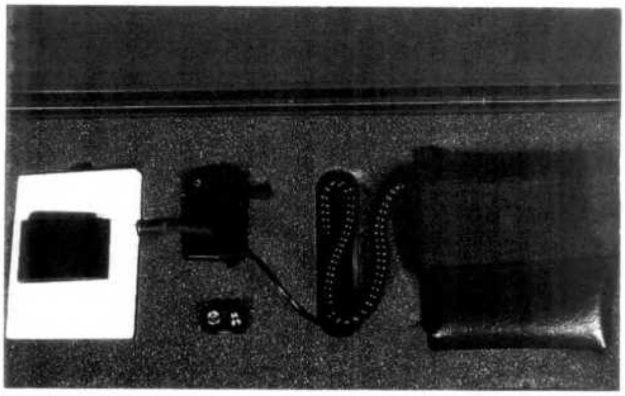

Figure 2. PELUT system displayed in a portable case.

tures involve the tibia, so the available safety data from more than 100,000 orthopaedic patients could be extrapolated to our use of comparable PEMF signals to treat leg ulcers. In addition, earlier *in vitro* studies employing the PEMF parameters described in this study have demonstrated measurable biological effects (N. Guzelsu *et al.*, unpublished data). All PELUT devices operated silently with no perceivable thermal, tactile or vibratory sensation. Neither patients nor investigators were able to discern any difference between active and placebo devices.

Efficacy parameters assessed at weeks 0, 4, 8 (and 12)

Wound surface area. The inner edge of the ulcer (border of epithelium) was traced on a Mylar sheet, and the surface area was calculated using computer image analysis by KGL, Inc. (Broomall, PA, U.S.A.). When this technique was attempted by multiple blinded investigators prior to initiation of the study, the degree of variation in wound area was less than 5%. This variation was primarily due to the thickness of the marker tip and to a lesser extent the difficulty in visualizing the ulcer border. Equipment used included: Magiscan 2B Image Analyzer (Joyce Loebl, Gateshead, U.K.), Dage Series 61 video camera (Dage-MTI, Inc., Michigan City, IN, U.S.A.), and software menu program developed by Joyce Loebl.

Wound depth. Wound depth, a secondary efficacy parameter, was measured at the deepest point using Digimatic Calipers (manufactured by Mitutoyo Corporation, Paramus, NJ, U.S.A.) or equivalent Digi-Met Electronic Calipers (Flexbar-Preisser Machine Corporation, Central Islip, NY, U.S.A.). Depth measurements obtained by these devices were significantly more accurate and reproducible than those obtained with a straight ruler as employed in earlier American studies.[27]

Granulation tissue. The quality and the quantity of the granulation tissue were clinically assessed by the investigator as the percentage of wound area with healthy bright pink or red-coloured tissue, or unhealthy grey or light pink-coloured tissue and necrotic tissue.

Clinical global assessment. The investigators' clinical global assessment of healing status was based on the eight-point scale outlined below: +4, healed; +3, marked improvement; +2, moderate improvement; +1, slight improvement; 0, no worse; −1, slightly worse; −2, moderately worse; −3, markedly worse. The primary factor in the clinical global assessment was wound area. Ulcer depth, appearance of granulation tissue and pain were also considered. Other factors including changes in oedema, stasis dermatitis and post-inflammatory hyperpigmentation were given lesser consideration.

Pain intensity. Patients reported pain intensity at the wound site on a four-point scale: 0, none; 1, mild; 2, moderate; 3, severe.

Statistical analysis

All analyses were performed with the Statistical Analysis System (SAS Institute Inc., Cary, NC, U.S.A.) on an IBM 3081 mainframe computer. The active vs. placebo differences were analysed for statistical significance by computing one-sided *P* values from analysis of variance (ANOVA) and from Kruskal–Wallis analysis of variance by ranks (rank-ANOVA).[28,29] The rank-ANOVA was included because it does not require the statistical assumptions of a Gaussian distribution or homogeneity of variances.

Results

Treatment with the active PELUT device resulted in statistically significant improvement in all efficacy parameters compared to treatment with the placebo device. At week 0 (baseline), there was no statistically significant difference between the two treatment groups with respect to any of the efficacy parameters. However, it is noteworthy that the ulcers in the placebo group were rated more painful at baseline, and the difference in this secondary efficacy parameter was marginally significant. Four patients (one active, three placebo) failed to complete the study, defaulting between days 28 and 38. One patient was lost to follow-up and could not be contacted by telephone or certified mail. The other defaulters cited personal reasons not related to adverse events or lack of progress during study participation.

Wound area

The week 0 and week 8 wound surface areas for the active and placebo groups are shown in Figure 3. The changes in wound surface area were a 47·1% decrease for active and 48·7% increase for placebo ($P<0{\cdot}0002$). For the patients who discontinued the study prior to day 42, week 8 wound area was determined by two methods: (i) estimation by linear extrapolation to day 56, or (ii) use of the last observed wound area in place of week 8 value.

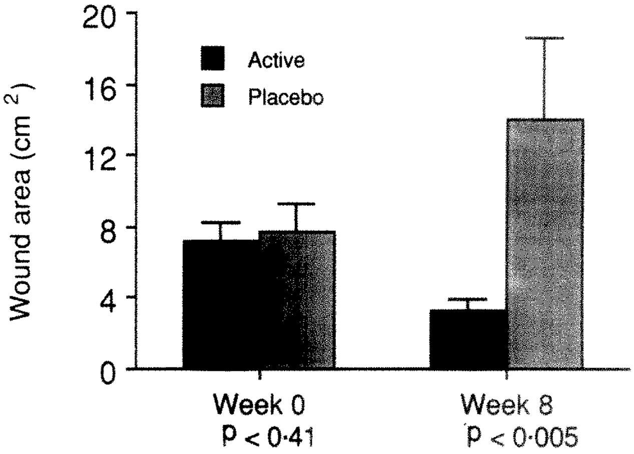

Figure 3. Wound surface area at baseline and after 8 weeks of treatment with PELUT or placebo device.

The results based on the last observed values were similar to those based on extrapolated week 8 values: the active group averaged a 47·7% decrease in wound surface area vs. a 42·3% increase in wound area for the placebo group ($P<0{\cdot}0002$). Change and per cent change in wound area were calculated for each patient in order to account for variability in wound size. In both active and placebo groups, there was no correlation between either the initial ulcer size or the type of ancillary treatment and the degree of subsequent change in wound area.

Wound depth

In the active group the average wound depth decreased from 0·24 ± 0·04 cm to 0·13 ± 0·02 cm. In the placebo group the average wound depth was 0·26 ± 0·01 cm at baseline and 0·25 ± 0·03 cm after treatment ($P<0{\cdot}04$) (Fig. 4).

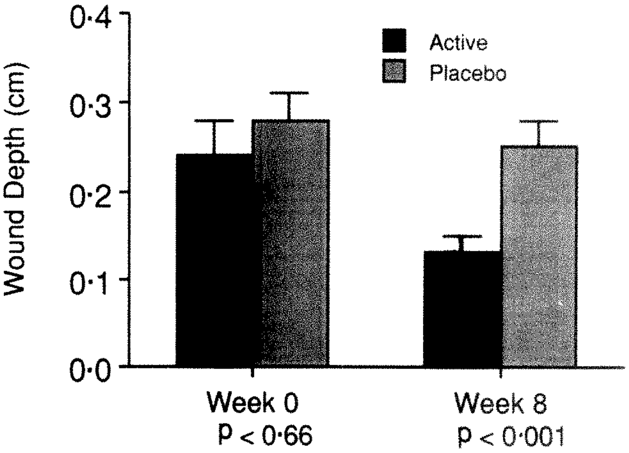

Figure 4. Wound depth at baseline and week 8.

Granulation tissue

In the active group the average percentage of healthy granulation tissue increased from 68·1±6·4% to 83·2±4·4%. In the placebo group the average percentage of healthy granulation tissue was 67·1±8·3% at week 0 and 67·5±7·7% at week 8 ($P<0·04$). There was a 14·1% decrease in unhealthy granulation tissue for the active group and 0% change for the placebo group ($P<0·04$).

Investigators' clinical global assessment at week 8

Fifty per cent of the active group healed or markedly improved compared with 0% of the placebo group. In contrast, 54% of the placebo group was rated worse compared with 0% of the active group ($P<0·001$).

Pain intensity

Wound site pain showed a score decrease of 0·61 (on the four-point scale mentioned previously) in the active group and a score decrease of 0·15 in the placebo group ($P<0·04$).

Twelve-week treatment group

Of the 12 patients who continued double-blind PELUT therapy to week 12, 11 patients were from the active group and only one patient was from the placebo group. At week 12, the active patients exhibited 66·3% decrease in wound surface area, representing further improvement from their 47·9% decrease in wound area at week 8. The placebo patient, who at week 8 had a 39·2% decrease in wound area, exhibited only a slight further decrease in wound area to 42·8% at week 12.

Adverse effects

There were no reports of patient complaints or adverse events attributable to the use of the PELUT device.

Discussion

The results of this study indicate that PEMF stimulation effectively and safely enhances the healing of recalcitrant venous stasis ulcers. Active PELUT therapy, 3 h daily for 8 weeks, led to a 47% decrease in wound area, a significant decrease in wound depth, 15% increase in healthy granulation tissue, and improved pain intensity scores. The patients who continued active treatment to 12 weeks exhibited further improvement (66% decrease in wound area). It is also noteworthy that half of the PEMF-stimulated ulcers healed or improved markedly, compared with none in the placebo group.

It is well known that venous leg ulcers may spontaneously improve or worsen. Ieran *et al.*, evaluating the effect of a unidirectional PEMF device on venous ulcers reported significant spontaneous healing in their control group (30% decrease in wound size over 90 days).[23] However, the control group in our study exhibited a 48% increase in wound area. It is possible that the ulcers in this study, because of stringent protocol inclusion–exclusion criteria and because the study sites were tertiary referral centres, were particularly recalcitrant. The relatively small number of patients enrolled in this clinical trial may have also contributed to the placebo effect, but our rigorous statistical analyses preclude this as a major factor. A recalcitrant ulcer in this study was defined as a stage II or III ulcer that had demonstrated poor healing in response to topical treatment for at least 4 weeks prior to enrollment in the study. In addition, wound size limitations and wound stability (<15% change in wound diameter and granulation tissue during 2 weeks prior to week 0) provided a homogeneous group of recalcitrant ulcers in this clinical trial. In the Ieran study, recalcitrance and wound stability were not protocol requirements. Although we cannot rule out a possible adverse effect of wearing the placebo device, this is unlikely given the light weight and portability of the device, as well as the improvement and absence of adverse events in the active group.

Whether PEMF stimulation facilitates ulcer healing by simply accelerating the normal healing process or by altering the mechanism of wound healing cannot be ascertained from this investigation. In a recent review of clinical applications of electric fields in soft tissue repair, Canaday and Lee concluded that electric or magnetic fields may accelerate wound healing only under circumstances in which the healing process is delayed or arrested, i.e. conditions of deficient or absent electrical current.[30] It is also of interest that to date, no healing enhancing agent has significantly altered the kinetics of normal healing.[30]

In vitro studies have demonstrated diverse cellular responses to PEMFs that may be relevant to wound healing. For example, migration of cultured fibroblasts and epithelial cells in perpendicular alignment to applied electrical fields is a well established phenomenon.[10,21,31] Stimulation with sinusoidal electromagnetic fields has been shown to induce differentiation of human skin fibroblasts and increase collagen and total protein synthesis.[19] PEMF stimulation employing physical parameters similar to those used in the present investigation resulted in enhanced proliferation of fibroblasts, as measured by increased DNA synthesis (N.Guzelsu *et al*., unpublished data). Increased angiogenesis in response to PEMFs is also thought to play a role in wound healing. Yen-Patton *et al.* reported that endothelial cells in culture grow at a higher rate and reorganize into three-dimensional vessel-like structures in the presence of a PEMF.[18] The specificity of the electromagnetic stimulus-response relationship is clearly demonstrated in the transcription studies by Goodman *et al.*[9] Exposure of human cultured cells (HL 60) to PEMFs with different characteristics resulted in signal-specific quantitative changes in RNA transcripts.[32] Despite the growing literature of the basic mechanisms underlying PEMF's biological effects, it is difficult to extrapolate such data to account for *in vivo* events. A systematic approach should be taken to characterize the 'windows' of PEMF parameters for cellular responses, as well as for complex *in vivo* events such as wound healing.

In light of recent controversy regarding the potential health hazards of environmental electromagnetic fields, the safety of PEMFs is addressed here. First of all, PEMFs were designed to induce electrical fields in tissue similar to those which normally result from electrokinetic events when tissues are mechanically deformed.[4,22] Second, more than 100,000 cases of non-union fractures and aseptic necrosis have been treated with PEMFs without any reported adverse effect.[6] Third, biological effects of PEMFs have been shown to arise within specific windows of stimulus parameters; therefore, we cannot extrapolate the effects of specified conditions to all PEMFs. Furthermore, the electrical field induced by a PEMF device is localized and its intensity decreases rapidly with increasing distance from the source. In addition, the energy carried by such low frequency PEMFs is thought to be too weak to break chemical bonds or have thermal effects.[9] Epidemiological data correlating environmental electromagnetic fields (power lines, electric blankets, video data terminals, etc.) with cancer, fertility and developmental problems have thus far been inconclusive.[22,33,34]

We conclude from this study that PELUT is an effective and safe adjunct to non-surgical management of recalcitrant venous leg ulcers. Additional studies are being initiated to confirm these results in a larger population and to evaluate the applicability of the PELUT system in the management of recalcitrant ulcers due to causes other than venous stasis.

Acknowledgment

We wish to thank John J.Frey, PhD, for his assistance in the statistical analysis of the data from this clinical trial. This study was funded in part by grants from Geomed, Inc. and the National Institutes of Health (Skin Disease Research Center Grant AR 39749).

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British Journal of Dermatology (1992) 127, 155–159.

Roxithromycin versus penicillin in the treatment of erysipelas in adults: a comparative study

P.BERNARD,* P.PLANTIN,† H.ROGER,‡ B.SASSOLAS,† E.VILLARET,§ V.LEGRAIN,¶
J.-C.ROUJEAU,** Y.REZVANI†† AND A.SCHEIMBERG††

**Department of Dermatology, CHU Dupuytren, Limoges, France*
†Department of Dermatology, CHU Morvan, Brest, France
‡Department of Dermatology, CHU Hotel-Dieu, Clermont-Ferrand, France
§Department of Dermatology, Hôpital Pellegrin, Bordeaux, France
¶Department of Dermatology, Hôpital des Enfants, Bordeaux, France
***Department of Dermatology, CHU Henri Mondor, Créteil, France*
††Laboratoires Roussel, Paris, France

Accepted for publication 6 April 1992

Summary A prospective, randomized, multicentre trial was conducted to evaluate the efficacy and safety of roxithromycin (150 mg b.i.d. orally) and penicillin (2·5 MU × 8 daily intravenously, then 6 MU daily orally) in the treatment of hospitalized adult patients with erysipelas. Seventy-two patients entered the study. Thirty-one patients in the roxithromycin group and 38 patients in the penicillin group completed the trial. The overall efficacy rates (cure without additional antibiotics) were 84% (26/31) in the roxithromycin group and 76% (29/38) in the penicillin group ($P=0{\cdot}43$). No side-effects were observed in the roxithromycin-treated patients whereas rashes occurred in two cases in the penicillin group, leading to exclusion from the study. Oral roxithromycin can thus be considered an effective and well-tolerated treatment for erysipelas in adult hospitalized patients.

Acute dermal and soft-tissue infections are commonly encountered in adults. Most cases may be clinically classified as erysipelas, i.e. acute plaque-like inflammation of the dermis and subcutaneous fat tissue that is often associated with fever, lymphadenopathy and leucocytosis.[1-3] Recent reports have emphasized the increasing incidence of erysipelas and a strikingly high prevalence of cases affecting the lower limb.[2,4,5] As erysipelas differs from acute cellulitis only in that it has well-defined margins,[1,2] it is often difficult, if not impossible, to clinically differentiate these two forms of cutaneous infection, particularly when they are localized on the lower limb. Moreover, they are both caused, in most cases, by *Streptococcus* organisms, especially *Streptococcus pyogenes*, as recently demonstrated by a combination of methods including conventional bacterial cultures, serology and direct immunofluorescence on skin biopsy specimens.[6] In this study, it was concluded that erysipelas and acute cellulitis in adults are two clinical aspects of the same infectious process, mostly due to streptococci.[6] Although treatment regimens vary widely, penicillin remains the reference treatment for erysipelas in adults.[2,3,5,7,8] However, side-effects of penicillin are not infrequent, and bioavailability in the skin is not well established. Roxithromycin is a new semi-synthetic macrolide with an antibacterial spectrum similar to that of erythromycin, but with improved pharmacokinetic properties that suggest its usefulness in the treatment of erysipelas.[9-11] The aim of the present study was to compare the efficacy and safety of roxithromycin and penicillin in the treatment of erysipelas in hospitalized adults.

Methods

Patients

The study was carried out in six dermatological departments in France between January 1989 and August 1990. All patients included were 15 years of age or older, and were hospitalized for acute, non-gangrenous dermal and soft-tissue infection. Signed informed consent was obtained from all patients. The diagnosis of erysipelas was made on clinical (cutaneous lesion characterized by erythema, increased warmth and

Correspondence: Dr P.Bernard, Hôpital Universitaire Dupuytren, 2 avenue Alexis Carrel, 87042 Limoges Cedex, France.

swelling with a plaque-like appearance; body temperature of 38°C or higher) and laboratory (WBC $>10 \times 10^9$/l) criteria. The presence of an elevated and well-defined margin of the cutaneous lesion was not required as a criterion.

Patients were excluded from the study for the following reasons: pregnancy; known allergy to macrolides or beta-lactams; known serious liver or renal disease; use of ergot alkaloids; antimicrobial treatment during the previous 7 days or use of other antibiotics concurrently; erysipelas involving the head; likelihood of another diagnosis, particularly necrotizing fasciitis; subcutaneous abscess and deep vein thrombosis of a lower leg.

Microbiological procedures

Conventional bacteriological cultures for aerobic and anaerobic bacteria were obtained from the patient's blood, from a swab of the punch biopsy site (see below), and from all cutaneous lesions (blister fluid, bordering ulceration, or other possible sites of entry). Swabs were plated on to 5% sheep-blood-agar plates within 24 h of collection. The blood-agar plates were incubated aerobically at 35°C and examined after 24 and 48 h. Beta-haemolytic colonies were screened for streptococcal group A, B, C, D and G using a latex agglutination test (BioMerieux, Lyon, France). *Staphylococcus aureus* was identified by the tube coagulase test and catalase production. When micro-organisms were isolated and identified, their antibiotic sensitivity profiles were subsequently determined by standard methods. In all but 14 cases biopsy specimens of involved skin were also tested for *Streptococcus* micro-organisms (groups A,C,D, and G) by direct immunofluorescence as previously reported.[6] The antistreptolysin 0 and antistreptodornase tests were performed at day 0 and day 15 of treatment.

Treatment

The patients were randomly allocated to two treatment groups. One group received oral roxithromycin 2 × 150 mg tablets per day. The other group received penicillin 2·5 MU every 3 h intravenously until apyrexial and then 3 × 2 MU tablets penicillin orally. Both antibiotics were continued until the patient had been apyrexial for at least 10 days. If abcesses formed they were surgically drained.

Evaluation

Clinical evaluation was performed before enrollment in the study, daily during the first week of treatment, and then at day 15 and day 30 after the start of treatment. At each clinical evaluation, erythema, oedema and pain were scored 0 (absent), + (moderate) or + + (marked), and the rectal temperature recorded. Laboratory tests including complete blood count, erythrocyte sedimentation rate and blood chemistry were performed on day 0, 7, 15 and 30 of treatment. Patients who showed no clinical improvement after 4 days of treatment, or whose local or general state worsened (particularly when the development of necrotizing fasciitis was suspected) left the trial at any time of treatment. They were considered as 'failures'.

Statistical analysis

Statistical comparisons between the two treatment groups were made with the following tests: Student's *t*-test, Fisher's exact test and χ^2 test (comparison of the two treatment groups and overall efficacy) and Log-rank test (delay of disappearance of symptoms).

Results

A total of 72 patients were enrolled in the study. After the exclusion of three patients receiving roxithromycin (two in whom the diagnosis of erysipelas was not confirmed, one with additional antimicrobial treatment), 31 patients in the roxithromycin group and 38 in the penicillin group were evaluated. The characteristics of the patients are shown in Table 1. There were no statistically significant differences between the two groups with respect to their demographic data, accompanying diseases, or clinical features of infection.

Table 1. Patients' characteristics

	Data of patients on	
Characteristics	Roxithromycin	Penicillin
No. of patients	31	38
Mean age (year) (range)	61 (19–92)	62 (20–92)
Sex (M/F)	19/12	19/19
Mean weight (kg) (range)	78 (45–120)	72 (41–125)
Immunosuppression	2	4
Non-steroidal anti-inflammatory drug intake	5	4
Duration of fever (days) prior to treatment (range)	2·6 (1–8)	2·4 (1–7)
Duration of local signs (days) prior to treatment (range)	3 (1–9)	4 (1–30)

Table 2. Bacteriological investigations in patients treated with roxithromycin ($n=31$) and penicillin ($n=38$)

Organisms	No. of isolates*	
	Roxithromycin	Penicillin
Streptococcus group A	16 (15)†	16 (14)
Streptococcus group G	5	5 (4)
Streptococcus group D	2	2 (1)
Streptococcus group B	1 (1)	—
Streptococcus group C	—	1
Staphylococcus aureus	12 (4)	10 (3)
Pseudomonas aeruginosa	2	6 (1)
Escherichia coli	1	2
Enterobacter cloacae	2 (1)	—
Klebsiella pneumoniae	1	—
Proteus mirabilis	—	12
None	5	11

* Detected by direct immunofluorescence (for streptococci only) and/or conventional cultures.

† Total no. positive (no. positive in lesional skin)

Table 3. Staphylococcal cause of erysipelas

Positive cultures for *Staphylococcus aureus*	No of patients on	
	Roxithromycin	Penicillin
In lesional skin (no. of patients)	4 (1)*	3 (1)
In portal of entry or blood cultures (no. of patients)	8 (4)	7 (1)
None	19	28

* Total no. positive (no. positive without evidence for a streptococcal cause).

Tables 2 and 3 summarize the microbiological data of the patients in the two treatment groups. All group A,C and G *Streptococcus* micro-organisms isolated by standard methods were susceptible to both penicillin and erythromycin. According to the conventional bacteriological cultures and/or the direct immunofluorescence data, a streptococcal cause could be determined in 20 patients from each group. Two patients in the roxithromycin group and nine patients in the penicillin group had only serological (raised antistreptolysin 0 and antistreptodornase titres) evidence for a streptococcal cause of their erysipelas. Thus, a streptococcal cause of erysipelas was considered as certain or probable in 22/31 (71%) patients in the roxithromycin group, and 29/38 (76%) patients in the penicillin group. Conversely, the remaining nine patients of each group did not have any laboratory evidence for a streptococcal cause. Non-streptococcal organisms were found in the majority (77%) of cases at suspected sites of entry (Table 2). *Staphylococcus aureus* was isolated from 22 patients (Tables 2 and 3), but was considered as pathogenic in only 12 patients (17%), i.e. when cultures grew from lesional skin (from a swab of the punch biopsy site) and/or when no evidence for a streptococcal infection was present. *Staphylococcus aureus* strains were resistant to penicillin in 83% (19/22) of cases, and to erythromycin and/or roxithromycin in only 32% (7/22).

The average duration of treatment was 13 days (range 2–29) for both antibiotics. In the case of penicillin, the average treatment duration was 6 days intravenously (range 2–17) followed by 7 days orally (range 0–21).

The overall efficacy rates (cure without additional antibiotics) were 84% in the roxithromycin group and 76% in the penicillin group, without statistically significant difference ($P=0{\cdot}43$). The clinical efficacy of both treatments is illustrated by the regression of the fever and local symptoms during the first week (Fig. 1). When the overall efficacy was analysed in subgroups of patients (i) with a streptococcal cause; (ii) with *Staphylococcus aureus* isolated from lesional skin or (iii) from sites of entry or with micro-organisms other than streptococci (including staphylococci), we found no statistically significant difference between the two treatment groups (Table 4).

No clinical adverse effects were seen in the patients treated with roxithromycin, but two cases in the penicil-

Table 4. Comparison of the efficacy of roxithromycin and penicillin in the 69 study patients

	Treatment		
	Roxithromycin	Penicillin	*P*
Overall efficacy (%)	84 (26/31)	76 (29/38)	NS
Patients with a streptococcal cause (%)	83 (19/22)	82 (23/28)	NS
Patients with *Staphylococcus aureus* isolated (%)	75 (9/12)	50 (5/10)	NS
Patients with organisms other than streptococci isolated (%)	83 (15/18)	71 (15/21)	NS

NS, not significant.

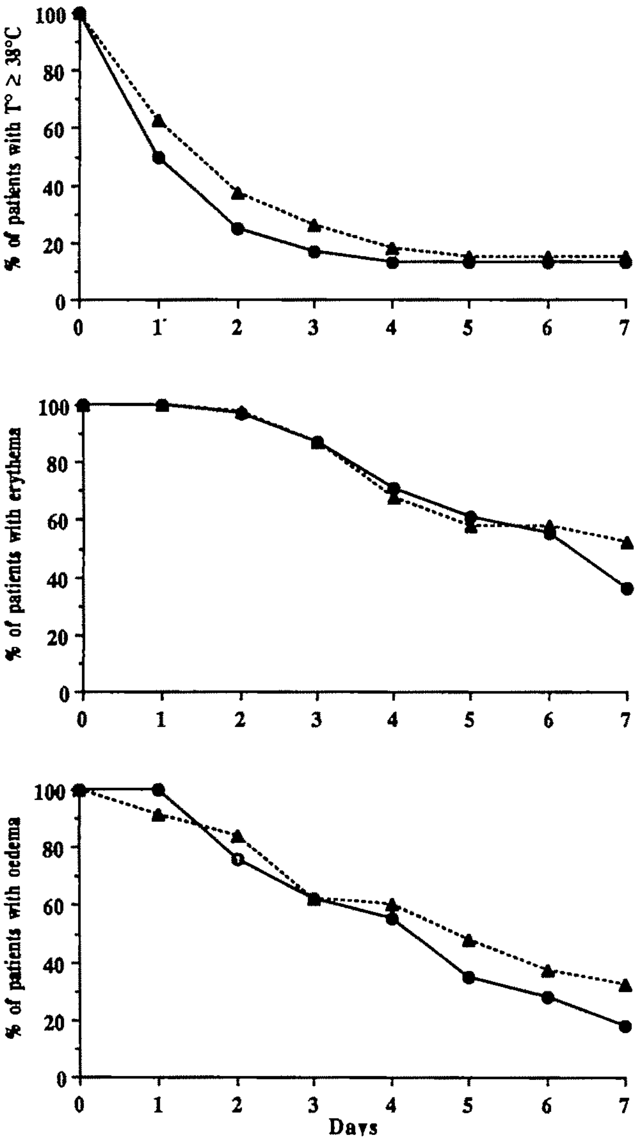

Figure 1. Time taken for resolution of fever, erythema and oedema in the two treatment groups. —●—, Roxithromycin; —▲— penicillin.

lin group developed rashes. No abnormalities in laboratory parameters were observed.

Discussion

It is striking that, to date, no prospective, randomized studies have been published that evaluate the therapeutic effects of penicillin, the most frequently recommended antibiotic in erysipelas and related acute dermal and soft-tissue infections. Penicillin treatment regimens for erysipelas and cellulitis are based upon retrospective studies and include intramuscular procaine penicillin[2] and intravenous or oral penicillin, all with variable administration schemes.[3,5,7] Combined treatment with cloxacillin has also been reported.[8] Recent studies have evaluated other antibiotics for the treatment of cellulitis, including cefotaxime, nafcillin, cefonicid, ciprofloxacin, ofloxacin and ceftriaxone.[12–15] However, in these studies, these antibiotics were not compared with standard penicillin treatment in erysipelas or acute cellulitis.

The results of this prospective, randomized and multicentre trial show that oral roxithromycin (daily dose 300 mg) is at least as effective as penicillin given initially intravenously (daily dose 20 MU daily), and then orally (6 MU daily). The overall efficacy obtained with roxithromycin is satisfactory when compared with penicillin (84 vs. 77%). However, it is possible that the number of patients did not allow detection of a difference between the two treatments ($P=0{\cdot}43$). The clinical efficacy of both treatments is well illustrated by the regression of fever and local signs (Fig. 1a–c), without any statistically significant difference between the two treatment groups (Log-rank test; $P=0{\cdot}442$, $P=0{\cdot}319$ and $P=0{\cdot}270$ for the comparison of the curves of temperature, erythema and oedema in both groups, respectively). It is noteworthy that fever generally disappears within 2 days of treatment, whereas local signs (erythema and oedema) persist after 5 days in more than 50% of cases.

A streptococcal cause of erysipelas was certain or probable in 71 and 76% of cases in the roxithromycin and penicillin group, respectively. The bacteriological data of this study are in complete agreement with another recent series from our group. The same direct immunofluorescence technique for the detection of streptococci in biopsy specimens of involved skin was used.[6] In contrast, the role of staphylococci in erysipelas and related acute dermal and soft-tissue infections remains difficult to determine. Staphylococci are more frequently present on normal skin than streptococci.[16] Staphylococci are thought to be either primarily responsible, or responsible for co-infection or super-infection of erysipelas.[17–19] In the present study, *Staphylococcus aureus* was considered responsible for the infection in only 12 patients (17%) who had no laboratory evidence for a streptococcal infection, and in whom bacteria were either cultured from lesional skin (swab from the punch biopsy site) or found at possible sites of entry. In patients with *Staphylococcus aureus* isolated from lesional skin or from sites of entry, the treatment efficacy rate was 75% in the roxithromycin group and 50% in the penicillin group. Although not significant, this difference may be due to the better skin bioavailability of roxithromycin and to its superior activity against *Staphylococcus aureus*[9,10] which was confirmed by the antibiotic sensitivity profiles in our study. The penicillin doses used in this trial were adequate, and were higher than in previous studies.[2,17,20] The question remains open as to whether antibiotics used for the treatment of erysipelas should have good antibacterial activity against *Staphylococcus aureus* to prevent co-infection or secondary infection (for example, abscess formation).

As the data reported in the present study suggest that oral roxithromycin is as safe and effective as penicillin administered by intravenous and oral routes in erysipelas, some practical and economic points may be considered. Oral roxithromycin may be employed as a first-line antibiotic for erysipelas in patients allergic to beta-lactams, such as penicillin. Furthermore, the development of oral therapy for this common disorder should lead to early discharge from hospital and therefore be more economical. It remains, however, necessary to hospitalize patients suffering from erysipelas initially because necrotizing fasciitis in its early stage cannot always be excluded.[8,17,21] As our study was carried out in hospitalized patients, further studies are necessary to assess the efficacy of oral roxithromycin in treating erysipelas for a 1–2 day period in hospital followed by further out-patient treatment.

Acknowledgments

We wish to thank Dr Conrad Hauser for critical assessment of the manuscript.

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British Journal of Dermatology (1992) **127**, 160–163.

Multifocal aplasia cutis congenita, distal limb hemimelia, and cutis marmorata telangiectatica in a patient with Adams–Oliver syndrome

K.BORK AND J.PFEIFLE
Department of Dermatology, University of Mainz, Germany

Accepted for publication 26 February 1992

Summary We describe an 18-month-old boy with multifocal scalp defects over the posterior parietal region combined with an underlying defect of the skull, left lower limb distal hemimelia and generalized cutis marmorata telangiectatica, consistent with a diagnosis of Adams–Oliver syndrome (aplasia cutis congenita with distal transverse limb defects).

In 1945 Adams and Oliver described eight members in three generations of a family with an association of congenital scalp defects and distal limb defects.[1] Since then, at least 11 families with this syndrome, apparently inherited as an autosomal dominant trait with variable expression, have been reported.[1-11] In addition, a number of sporadic cases have been described.[12-17] To date, more than 80 affected individuals with the complete or incomplete form of Adams–Oliver syndrome have been reported. However, the syndrome has so far received little attention in the dermatological literature.[9]

Case report

An 18-month-old male infant was admitted to the dermatology department of the University of Mainz in December 1990. He was the youngest of nine siblings of a non-consanguineous German–Russian immigrant family. He was the product of a normal full-term pregnancy. There was no history of maternal infection or drug ingestion during the pregnancy. Her previous eight pregnancies had all been normal. At the time of the patient's conception the mother was 38 and the father 39 years old.

At birth the boy was noted to have circumscribed ulcerated areas on the scalp and a defect of the left leg. His subsequent development was normal.

Examination revealed a patch of cicatricial alopecia over the vertex and the parietal region, with three ulcerated areas of different sizes (Fig. 1), covered with haemorrhagic crusts. A skull X-ray showed an open anterior fontanelle, 10 × 10 cm in size (normally 2 × 2 cm at this age). The posterior fontanelle was closed. Head circumference was normal and an EEG showed no abnormality. There was a defect of the distal part of the left leg (Fig. 2). An additional finding was generalized cutis marmorata telangiectatica congenita (Fig. 3) including the scalp (Fig. 1), but otherwise the patient was normal, and there were no abnormalities of the toes or fingers on the intact limbs. Based upon these features, a diagnosis of Adams–Oliver syndrome was made.

The scalp lesions were treated with topical antibiotic creams. They became smaller within 3 months of initial presentation, and further topical therapy was stopped. The patient was fitted with a lower-limb prosthesis.

None of his siblings, the parents, grandparents, or other relatives were affected by either aplasia cutis congenita, limb defects, or cutis marmorata.

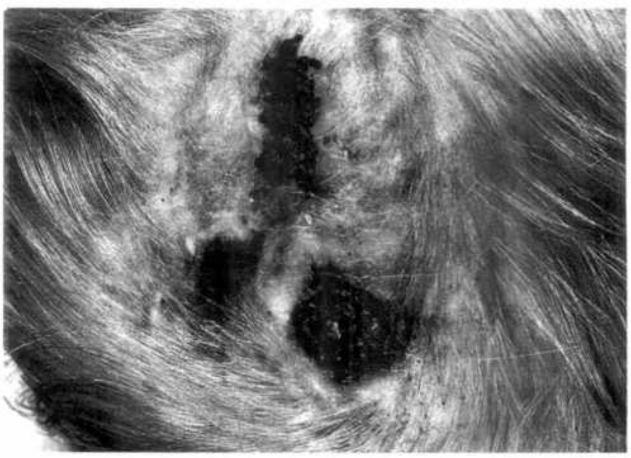

Figure 1. Aplasia cutis congenita, showing three ulcerated areas over the vertex covered with haemorrhagic crusts.

Correspondence: Professor Dr Konrad Bork, Department of Dermatology, University of Mainz, Langenbeckstraße 1, 6500 Mainz, Germany.

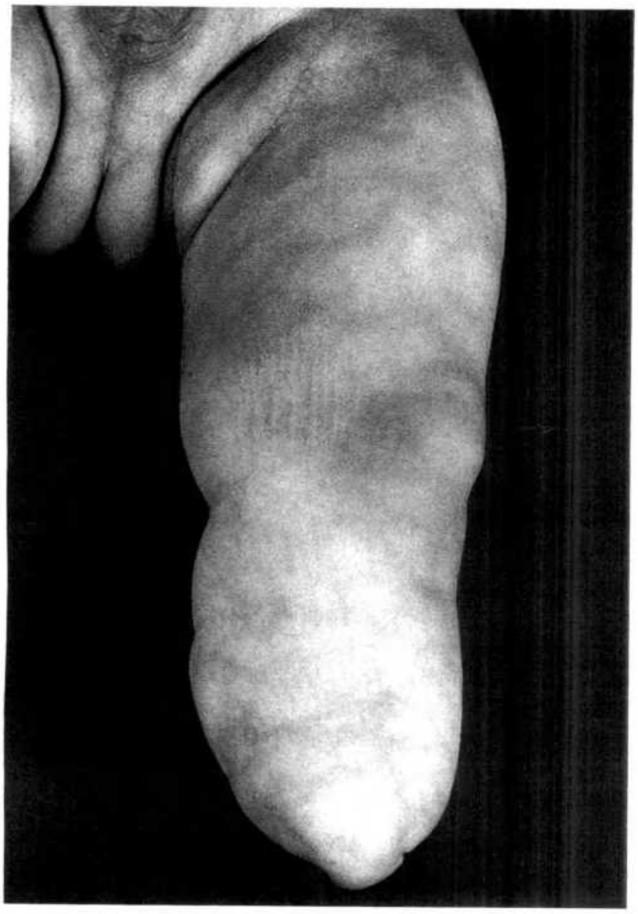

Figure 2. Transverse defect of the lower part of the left leg. Cutis marmorata telangiectatica is also visible.

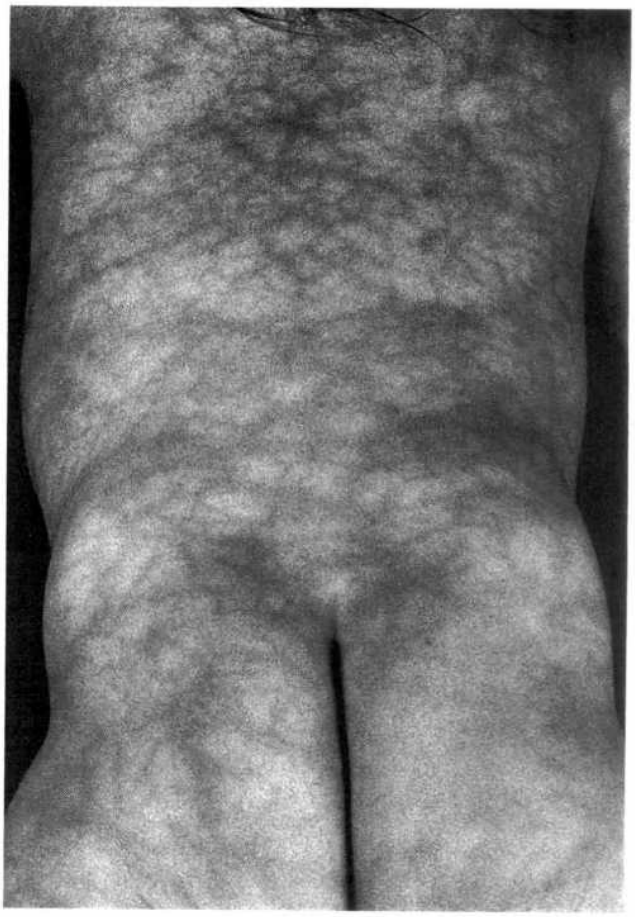

Figure 3. Generalized cutis marmorata telangiectatica congenita.

Discussion

Features of the Adams–Oliver syndrome include aplasia cutis congenita, distal limb anomalies and, less frequently, cutis marmorata telangiectatica congenita (Table 1).

Table 1. Main abnormalities in Adams–Oliver syndrome

Growth	Mild growth deficiency (third to tenth percentile)
Scalp	Aplasia cutis congenita over posterior parietal region with or without an underlying defect of bone. Tortuous veins over posterior scalp
Limbs	Variable degrees of terminal transverse defects including those of lower legs, feet, hands, fingers, toes and/or distal phalanges. Short fingers. Small toenails
Skin	Cutis marmorata telangiectatica

Aplasia cutis congenita is an uncommon disorder which usually occurs as an isolated condition. Most cases are sporadic, although familial cases have been reported as either an autosomal dominant or recessive trait.[18-21] The lesions heal over a period of months, leaving an atrophic or hypertrophic scar without hair. In some cases, aplasia cutis is associated with other congenital disorders including congenital heart disease,[22] tracheo-oesophageal fistula,[18] cleft lip and palate,[23] double uterus,[24] spinal dysraphism,[25] and cerebral disorders.[23,26-28] An association with distal limb abnormalities of varying degrees is most frequently observed and is now termed Adams–Oliver syndrome. Aplasia cutis as part of this syndrome does not seem to differ in any respect from isolated cases of aplasia cutis congenita, although healing of multiple defects, as in our patient, appears to be more protracted. The pathogenesis of aplasia cutis congenita is not known.

Abnormalities of the extremities in Adams–Oliver syndrome affect limbs, toes, fingers and nails (Table 2), the lower limbs being affected more than the upper

Table 2. Distribution of limb defects

Type of defect	Number of cases in the literature with these abnormalities
Hand	
Short fingers	17
Absent phalanges	7
Absent digits	9
Absent hand	3
Syndactyly	6
Feet	
Small toenails	1
Short toes	14
Absent distal phalanges	11
Absent toes	9
Absent feet	4
Absent lower leg	2
Unspecified	2
Syndactyly	4
Widely spaced 1st and 2nd toes	6
Club-foot	6

limbs. Limb involvement is usually asymmetrical. Although a variety of distal limb defects have been reported in Adams–Oliver syndrome, the type of limb defect seems to be specific for each family and there is no significant intrafamilial variation in the general category of limb defect.

Cutis marmorata telangiectatica congenita is an inconstant feature of the syndrome, occurring in about 25% of patients. Cutis marmorata congenita is observed much more frequently as an isolated phenomenon of unknown aetiology. However, it may be associated with other congenital abnormalities such as congenital heart defects.[29,30]

Not all patients show the complete form of Adams–Oliver syndrome. The association of transverse limb defects and cutis marmorata congenita without aplasia cutis has been reported.[31,32]

Many of the reported cases of Adams–Oliver syndrome occurred sporadically. In the majority of familial cases, the inheritance pattern was most consistent with that of an autosomal dominant gene with reduced penetrance and extremely variable expression. In two reports, however, an autosomal recessive inheritance was assumed.[33,34] Our patient has the clinical features of Adams–Oliver syndrome with congenital scalp/skull and limb anomalies associated with cutis marmorata congenita. It is a sporadic case, because the parents and other relatives are normal.

Distal limb hemimelia has until now been observed in only two patients, namely one of the patients originally described by Adams and Oliver and patient No. 6 reported by Sybert.[9] All other patients have shown anomalies of fingers and toes or, in some cases (Table 2), absence of a hand or a foot.

The delayed healing of the aplasia cutis in our patient is unusual. Such delayed healing has not been reported in other patients with Adams–Oliver syndrome or with cutis marmorata telangiectatica congenita associated with Adams–Oliver syndrome. We assume that locally impaired circulation due to cutis marmorata on the scalp explains the delayed healing in our patient.

The aetiology and pathogenesis of Adams–Oliver syndrome and of aplasia cutis congenita are not known. The familial occurrence and the association with other congenital defects suggest that it is not due to external influences or persistent amniotic bands. A common pathogenesis for both aplasia cutis over the vertex and distal limb defects could be a vascular disruptive mechanism. It has been suggested that local impairment of circulation or impaired vascular development due to rapid brain growth and subsequent tension and stretching of embryonic skin may be responsible for aplasia cutis congenita.[35] There is also some evidence for a vascular disruptive pathogenesis of distal transverse limb defects.[16,36] The association of cutis marmorata telangiectatica and, in some patients, dilated scalp veins and other vascular malformations,[26] may be a further sign of a vascular disorder as an aetiological factor in the development of aplasia cutis congenita and of Adams–Oliver syndrome.

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British Journal of Dermatology (1992) **127**, 164–167.

Extensive and fatal basal cell carcinoma: a report of three cases

C.B.KO, S.WALTON AND K.KECZKES
Department of Dermatology, Hull Royal Infirmary, Hull, U.K.

Accepted for publication 10 March 1992

Summary Three cases of basal cell carcinoma (BCC) with extensive invasion are described. The first two patients had meningeal and cerebral involvement with exposure of their dural meninges following full thickness skull erosion. The third patient had bilateral orbital and optic nerve involvement resulting in complete blindness. All three patients subsequently died from their disease.

Basal cell carcinoma is the most common malignant tumour of the skin, accounting for 70–75% of all skin tumours.[1] It normally spreads by local invasion, but metastasis has been reported.[1,2] In most cases treatment by surgical excision, radiotherapy, cryotherapy, or curettage and electrodessication can achieve a cure rate of over 90%.[3–6] Most patients present with early lesions, but in some cases delay in diagnosis may result in extensive invasion with a fatal outcome.

Case reports

Case 1

A 61-year-old reclusive, retired labourer was referred urgently to our department after his family doctor was informed by his worried relatives about a massive, foul-smelling, ulcerated lesion on the right half of his head. The lesion had increased in size relentlessly over a period of 6 years and the patient had been trying to hide it from others by covering the affected area with clothing. According to his relatives, he had always refused to seek medical advice due to a lifelong phobia of doctors.

Examination revealed an anxious man who had an extensive necrotic foul-smelling lesion over the right half of his scalp, temple and face, measuring about 17 × 12 cm (Fig. 1). The lesion had eroded into his right orbit, exposed the eyeball, and paralysed all the extra-ocular muscles. He had lost the sight in his right eye completely and there was partial loss of sight in the left eye. The tumour had also eroded his right temporal bone, exposing a pulsatile dural membrane. Clinically there was no evidence of lymph node involvement. There was weakness of the muscles of dorsiflexion on the left foot, but otherwise clinical examination was normal.

Correspondence: Dr. K.Keczkes, Hull Royal Infirmary, Anlaby Road, Hull HU3 2JZ, U.K.

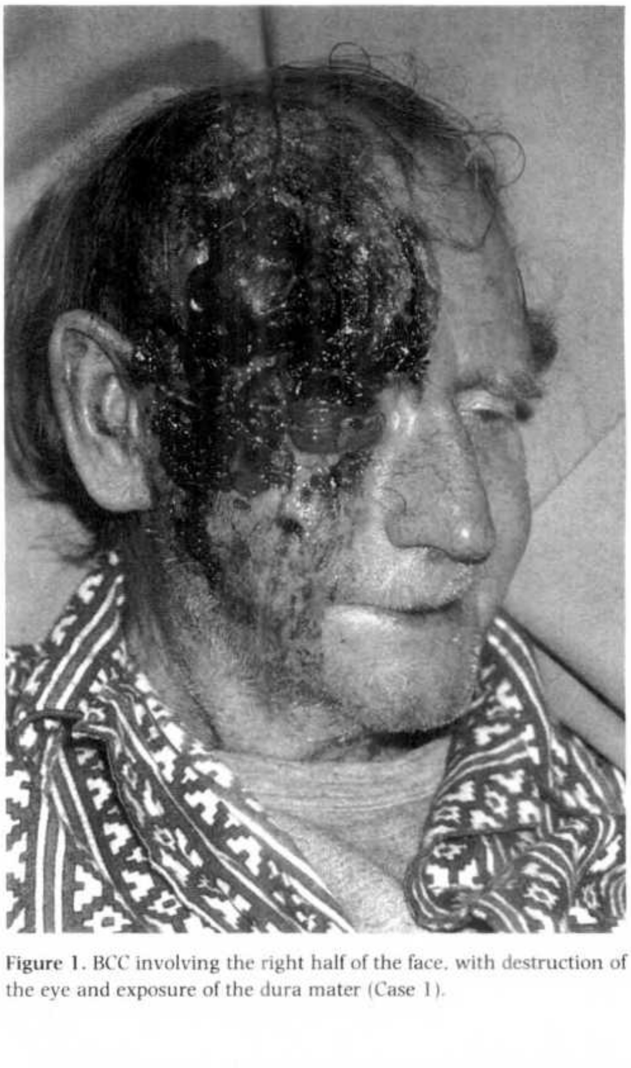

Figure 1. BCC involving the right half of the face, with destruction of the eye and exposure of the dura mater (Case 1).

A wedge biopsy taken from the massive lesion on his head showed the histology of a solid type of BCC. Computerized tomographic (CT) scanning of his head revealed vault destruction, invasion of the tumour into the supra-orbital plate and right frontal sinus, right and

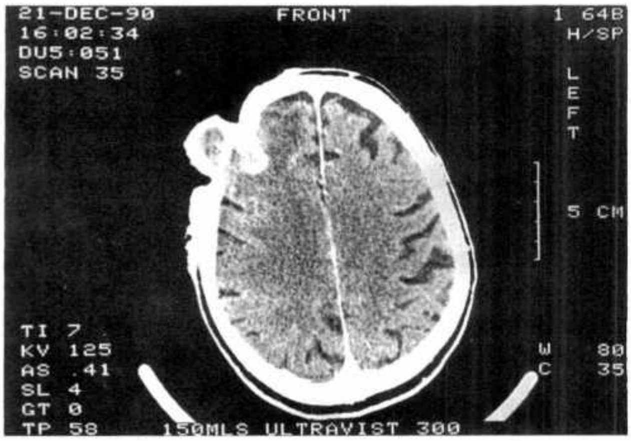

Figure 2. CT scan revealing destruction of the temporal bone and prolapsing dura mater and brain tissue (Case 1).

left anterior fossae, ethmoids, maxilla, right lateral ventricle, and skull base, and extension to the contralateral orbital region. The right frontal cerebral tissue was infiltrated and there was distortion of the frontal horn of the right lateral ventricle (Fig. 2). CT scan of his chest was normal, as were his full blood count and serum biochemical profile. He was assessed by a plastic surgeon, neurosurgeon and radiotherapist, all of whom felt that his tumour was too extensive and advanced for any intervention to be feasible. He was, therefore, treated conservatively with topical antiseptic wound care. His condition gradually deteriorated, and he died 3 weeks after admission.

Case 2

A 70-year-old woman presented with a large ulcerated lesion involving her anterior scalp, forehead, glabella, eyelids, and nose (Fig. 3). The lesion had been present for approximately 7 years, during which time it progressively increased in size. Eventually her relatives became concerned and contacted her family doctor, who promptly referred her to our department. Her reason for not seeking medical advice earlier was because she had been 'too busy' caring for her disabled husband until he died.

Clinical examination revealed a large, ulcerated BCC measuring about 20 × 15 cm, extending from her anterior scalp to her eyes and nose, and across her forehead. In the centre of the lesion there was a soft, rounded mass about 8 cm in diameter (Fig. 3). This was later found to be exposed dural membrane resulting from full thickness erosion of the skull by the basal cell carcinoma, although it was not initially recognized as such because of infiltration by tumour, and secondary infection. Surprisingly, her general condition was otherwise good and there were no clinical signs of meningism or neurological deficit. A wedge biopsy of the lesion was taken which not only confirmed the diagnosis of BCC, but also showed dural membrane heavily infiltrated by carcinoma, and the presence of brain tissue. She was treated with a course of radiotherapy, followed by total excision of the lesion combined with a dural graft, pedicle scalp flap and skin graft. At operation there was no obvious macroscopic invasion of the brain. Postoperatively she made an uneventful recovery and did not show any neurological deficit.

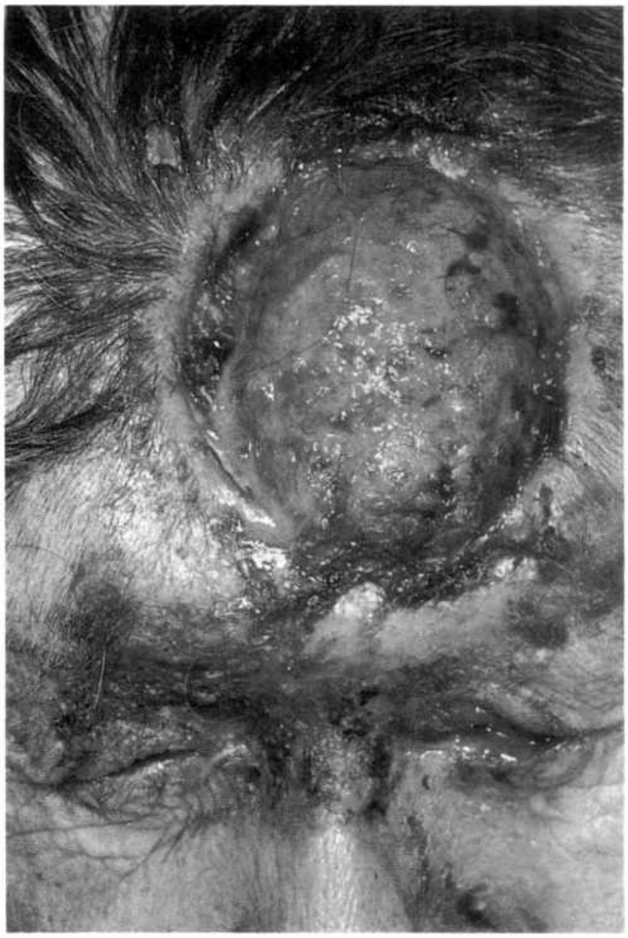

Figure 3. Close-up of exposed dura mater/brain through BCC on the forehead (Case 2).

Five years later she was re-admitted with recurrence of BCC at the same site. The dural graft had become infected and she had developed left proptosis associated with bleeding. A CT scan of her head revealed cerebral oedema and infiltration of the BCC into the dural graft and frontal cerebrum. She was treated conservatively with systemic antibiotics, oral steroids for her cerebral oedema, and topical antiseptics. Over the next 10 months her condition gradually deteriorated, she developed epileptic seizures, and eventually died from encephalitis.

Case 3

An 82-year-old woman was referred with a large BCC over the glabellar area which had destroyed the upper half of her nose and extended half way across both her upper and lower eyelids (Fig. 4). The lesion had first appeared 15 years previously and had progressively increased in size. She had otherwise been in good health and had not visited her family doctor for a long time. Her reason for not seeking medical advice earlier was that the lesion was painless and she had been 'too busy' looking after other people.

Her eyesight and fundi were normal, and there were no localizing neurological signs. She was reluctant to have any treatment, but eventually agreed. She underwent plastic surgery to excise as much tumour tissue as possible, and this included removal of nasal bones, nasal turbinates and the inner angles of the eyelids of both eyes, followed by skin grafting. Six months later she developed a local recurrence over the right inner canthus and this was re-excised. However, a year later, extensive recurrence was found over the grafted area. This was treated by radiotherapy without success. Over the next 5 years the tumour progressed relentlessly to involve the rest of the face and scalp, and invaded both orbits, causing blindness. She was admitted on several occasions for management of uncontrolled bleeding from the tumour, which had also become heavily infected, and she eventually died in hospital.

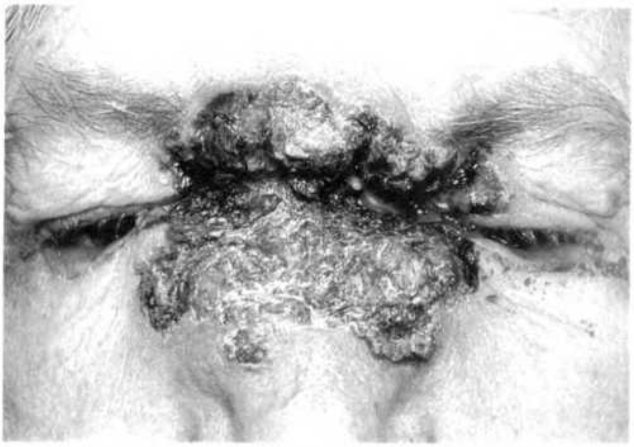

Figure 4. Destruction of the upper half of the face (Case 3).

Discussion

Basal cell carcinoma is the most common tumour affecting fair-skinned people.[7,8] The incidence of BCC is increasing,[9] and varies from 20 per 100,000 population per annum in Germany, about 110 per 100,000 per annum in the United States,[10] to as high as 1560 per 100,000 per annum in the northern part of Australia.[9] The majority (over 90%) of BCCs are cured by surgery, radiotherapy or other treatment modalities such as cryotherapy and curettage.[6] Such high cure rates can induce complacency and a belief that BCC is a trivial disease, and not potentially life-threatening. These three cases remind us of its aggressive and destructive nature, if neglected.

Nearly two-thirds of BCCs occur on the head.[11] Our cases demonstrate that one should be cautious when taking a biopsy from an enormous BCC on the head, especially when a large mass is present, as this could be exposed dural membrane/brain, recognition of which may be difficult because of heavy infiltration by the tumour and secondary bacterial infection. In Cases 1 and 2 we were surprised by the absence of any clinical signs of meningism despite evidence of bacterial infection and direct tumour invasion of the dura mater. Both patients also had invasion of BCC into the brain. This is unusual,[12] although a number of cases were cited by Rubenstein.[13] A case of meningeal carcinomatosis due to BCC has also been reported by Soffer *et al.*[14]

The management of extensive facial BCCs, such as these, is a challenge. No treatment was thought to be feasible for Case 1. It is of interest that in Case 2, who had radiotherapy prior to surgery, clinical recurrence of the BCC occurred 5 years after surgical excision, whereas in Case 3, who did not have prior radiotherapy, clinical recurrence occurred 6 months after surgery. Although both patients died about 5 years later, late recurrence in Case 2 had given her a better quality of life in the intervening period. Further studies may prove valuable in determining the benefit of radiotherapy prior to surgery for extensive BCCs, although radiotherapy alone is said to be ineffective once osseous invasion has occurred.[15] Hartman *et al.*,[16] however, reported a case of bony metastases from BCC which responded well to radiotherapy, with long-term survival. An aggressive surgical approach by ablative craniofacial composite orbitectomies was reported by Rosen[15] as having been effective in four patients with periorbital BCC invading the orbit and ethmoidal sinus. Chemosurgery, formerly by Mohs' 'fixed tissue' technique,[17,18] and subsequently with the 'fresh tissue' technique,[19] is probably the most effective treatment for very extensive BCC.

Oral retinoids may prove useful in the management of BCCs which are not amenable to surgery or radiotherapy. Jones *et al.*[20] reported a case of superficial multifocal BCC of the breast with a diameter of 6–7 cm which resolved completely following a 3-month course of etretinate at a dose of 25 mg t.d.s. Peck[21] also reported good results in 11 patients with multiple BCCs treated with isotretinoin. Immunotherapy, using agents such as interferons, has also been shown to be capable of achieving a cure rate of from 25% to as high as 100%.[22–25] This treatment may be useful for BCCs which are not suitable for surgery or radiotherapy. Combination cisplatin chemotherapy may also have a place in the treatment of BCCs when other modalities are impractical. A case of a large BCC, with biopsy-proven complete response to cisplatin-combination therapy, was reported by Dickie and Pratt,[26] while Guthrie *et al.*[27] and Coker *et al.*[28] have also reported cases of BCC responding favourably to other regimes of cisplatin-combined chemotherapy.

We were astonished to find neglected BCCs of such severity in the United Kingdom. Despite being the commonest form of malignant skin tumour, public awareness of BCC is lacking in comparison with malignant melanoma. In Cases 2 and 3 lack of awareness of the serious nature of their disease by the patients and their relatives appeared to be the main reason for their late referral. This report serves as a reminder that fatalities due to BCC can occur if the tumour is neglected.

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An acantholytic dyskeratotic epidermal naevus with other features of Darier's disease on the same side of the body

C.S.MUNRO AND N.H.COX

Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne, U.K.
**Department of Dermatology, Cumberland Infirmary, Carlisle, U.K.*

Accepted for publication 20 March 1992

Summary Many epidermal naevi with the histology of Darier's disease have been reported. In the absence of associated features of Darier's disease, they cannot be assumed to have a common pathogenesis with it, and it has been suggested that they are better classified as acantholytic dyskeratotic epidermal naevi rather than naevoid Darier's disease. We describe a patient with such a naevus who had typical nail and palmar changes of Darier's disease on the same side of the body. We suggest that in at least some cases the naevus has the same genetic defect(s) as generalized Darier's disease, and discuss the possibility that a patient with such a naevus could occasionally transmit Darier's disease to an offspring.

Darier's disease (keratosis follicularis) in a localized 'zosteriform' pattern was first reported by Kreibich in 1906,[1] and in 1933 Cockayne stated that in 10% of cases of Darier's disease the lesions had a 'linear distribution like a systematized naevus'.[2] Since then, many more unilateral, linear or zosteriform epidermal lesions with the clinical and histological characteristics of Darier's disease have been reported.[3–14] Starink and Woerdeman[3] reviewed 32 cases reported between 1933 and 1981, and discussed the relationship between 'localized keratosis follicularis' and Darier's disease proper. They concluded that, in view of the lack of family history, the occurrence of cases in infancy,[3,13] and the absence of other features of Darier's disease in any reported case, it is preferable to describe the localized lesions as acantholytic dyskeratotic epidermal naevi rather than localized Darier's disease. The question of whether the cellular defect(s) in the naevi are the same as those which cause Darier's disease proper remains unresolved. We describe a patient in whom this appears to be the case, as the typical rash of Darier's disease, in a unilateral naevoid distribution, was accompanied by other cutaneous features of the disorder on the same side of the body.

Case report

A 22-year-old nurse presented with a 10-year history of a rash affecting the left side of her body. She had noticed abnormal nails on the left hand since the age of 9. The rash had periodically been exacerbated by sun exposure. Neither parent was affected, and there was no other family history of skin disease. On examination she had groups of the typical brownish keratotic papules of Darier's disease, which were confined to the left side of the body, and were present on the chest, abdomen, back, neck and axilla. The lesions on her back were arranged in a whorled band over the left scapula, and extended across the midline for a short distance (Fig. 1a), but on the anterior trunk the lesions stopped at the midline. In the axilla the lesions were clearly arranged in streaks (Fig. 1b). In the left third and fourth fingernails (Fig. 2) and left great toenail there were red streaks terminating in notches. Small keratoses and pits were present in the skin ridges on her left hand, and on the dorsum of that hand there were numerous pinpoint acanthomas. None of these features of Darier's disease was present on the right side of her body. There were no oral lesions. Histology of a keratotic papule from the left chest showed acantholytic clefts, hyperkeratosis, and dyskeratosis with corps ronds and grains, and was typical of Darier's disease (Fig. 3).

Correspondence: Dr C.S.Munro, Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K.

Discussion

It has been proposed that localized lesions displaying the clinical and histological features of Darier's disease should be regarded as acantholytic dyskeratotic epidermal naevi and not as Darier's disease of limited extent.[3] This conclusion was reached because of the lack of other

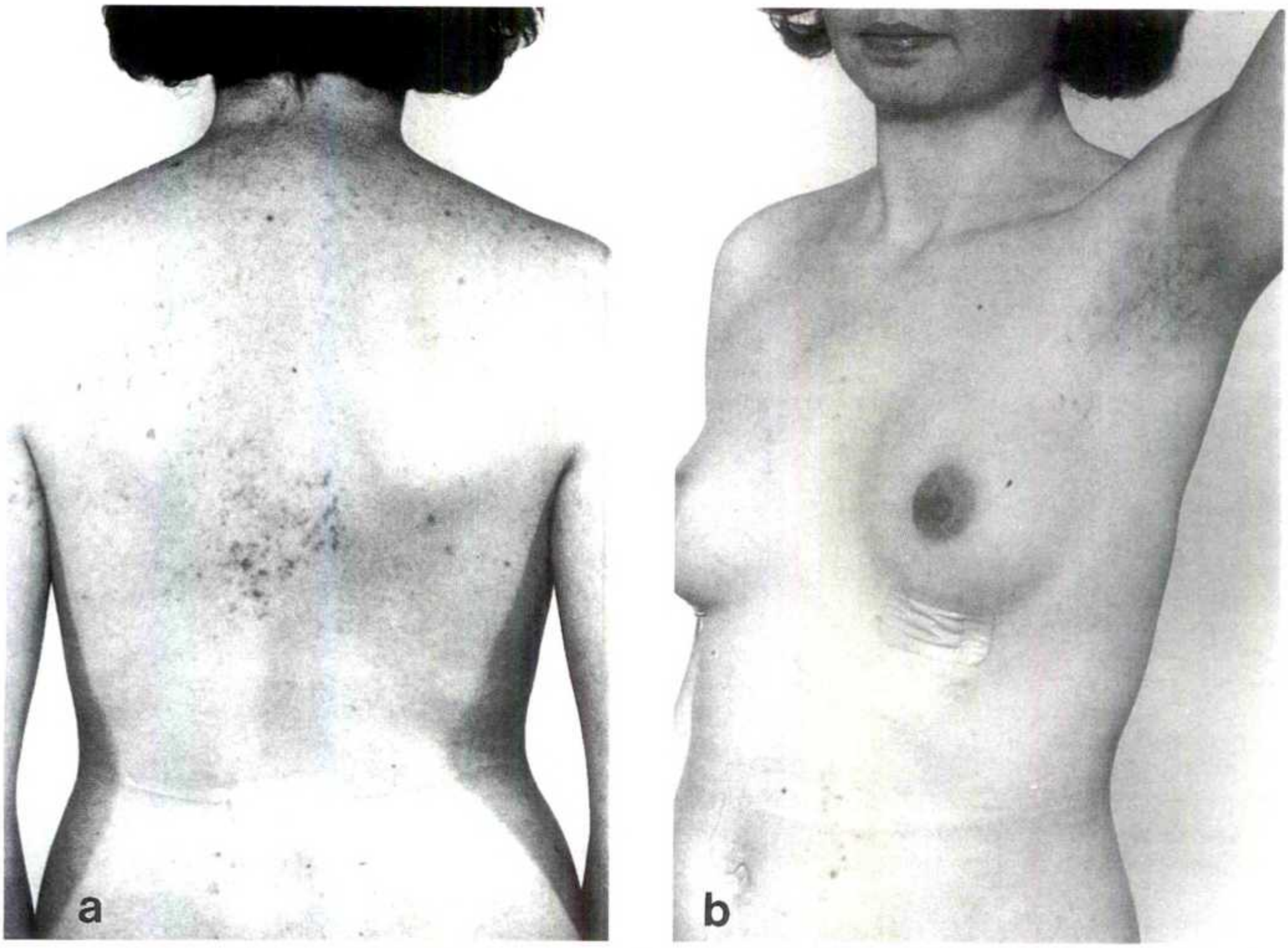

Figure 1. (a) Keratotic papules on the left upper back, in a whorled linear distribution consistent with Blaschko's lines. (b) Similar papules present in streaks in the left axilla and under the left breast.

features of the disease, and in the belief that these localized lesions never occur within families with Darier's disease. While the latter belief may be erroneous (see below), it remains most likely that such naevi are sporadic events, due to genetic mosaicism arising from a mutation in early embryogenesis.[15] In the absence of other features of Darier's disease, it cannot be assumed that the mutation(s) producing acantholytic dyskeratosis are the same as those which cause the generalized disorder.

However, other evidence favours the theory that at least some acantholytic dyskeratotic epidermal naevi are indeed localized Darier's disease. In addition to the clinical and histological appearances, the electron microscopic features are the same.[3] Unlike most epidermal naevi, the majority of cases have their onset in the second and third decades, or later,[3] and many display photoaggravation.[3,14] Our patient, who shows all the cutaneous features of Darier's disease in a restricted distribution, supports this argument. The pattern of lesions in the axilla and on the back is clearly naevoid, and follows the lines of Blaschko;[16] lesions slightly crossing the midline on the back are consistent with this pattern. The accompanying features of Darier's disease are strictly unilateral, and are not present on all digits. The typical nail and palmar changes of Darier's disease and the minute acanthomas which are found in this condition[17] are modest in extent, but indisputable.

Our case is rare or unique amongst patients with 'localized keratosis follicularis'. Of 42 previous cases in which we have examined the original reports,[1,3-14] and five additional patients known to our department, only one appears to have had other features of Darier's disease. This case, reported by Zamora Martín *et al.*,[9] differs from ours in that the nail changes and acanthomas on the hands were bilateral, and the skin lesions illustrated do not appear naevoid in distribution. These changes may therefore simply represent a localized rash

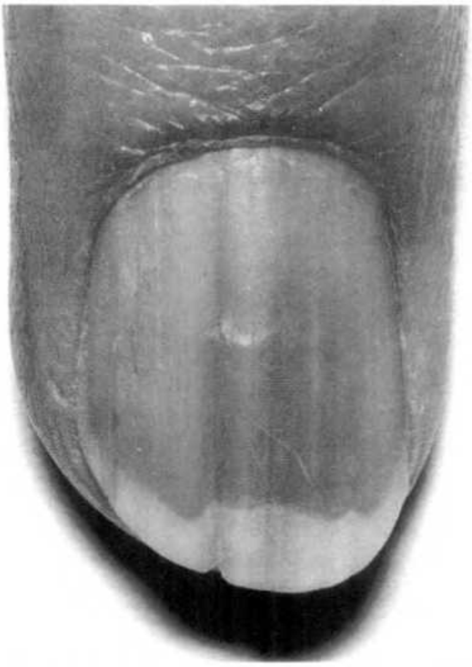

Figure 2. Typical streaks and distal notches of Darier's disease on the left middle fingernail.

in a patient with Darier's disease. The lack of associated features in all the other reported unilateral Darier's disease-like naevi may be due to non-involvement of the epidermal regions in which they arise. However, it is surprising that associated features have not been noted before if the pathogenesis of naevi is always identical to that of Darier's disease, and suggests that this is true of only a proportion of cases.

Alternative evidence for the identity of the genetic defect in some patients would be the birth of a child with Darier's disease to a parent with an acantholytic dyskeratotic naevus. In an analogous situation,[18] there are several cases in which parents, of either sex, with a linear naevus showing epidermolytic hyperkeratosis histologically, had children with generalized bullous ichthyosiform erythroderma of the same histological characteristics.[19] Our patient was concerned that, if her unilateral defect involved her left ovary, she might bear an affected child.

A somatic mutation giving rise to the anomaly and also affecting the gametes could explain naevoid Darier's disease occurring within kindreds, although naevoid lesions would be expected only in the progenitor.[15] There are no recent reports of this having occurred, but in his 1933 review of Darier's disease Cockayne[2] records that in each of four affected families, zosteriform lesions were present in one member. The relationship of the patients with naevoid disease to others affected is not stated. Moreover, it has been claimed[12] that in some familial cases the distribution of the bilateral rash can partially resemble a systematized naevus. In Darier's disease there is often no family history, and examining the parents of 'new' cases may potentially reveal the presence of acantholytic epidermal naevi. Conversely, patients with acantholytic dyskeratotic naevi may have a small but real chance of bearing a child with a generalized dyskeratosis.

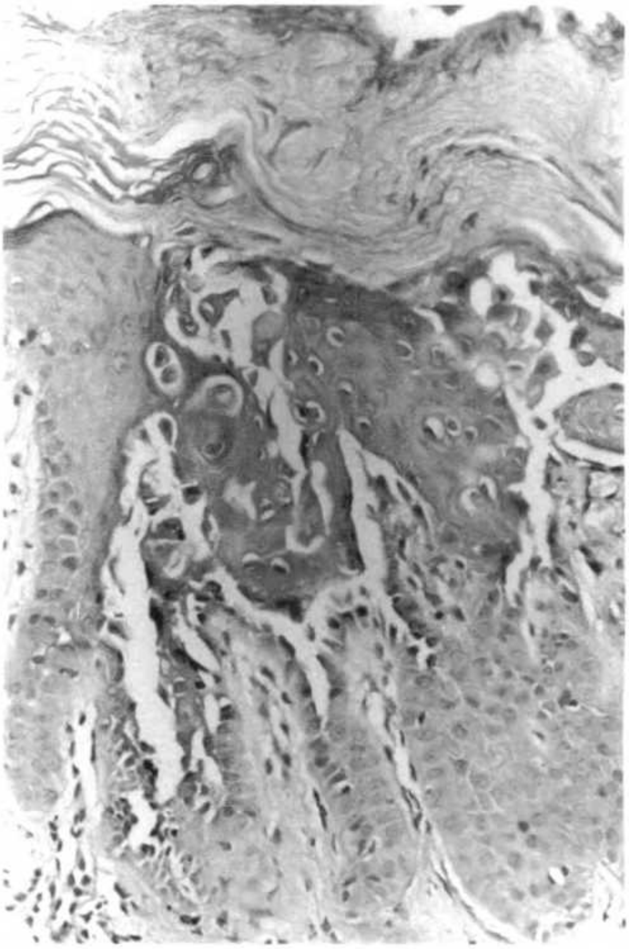

Figure 3. Histology of a papule showing suprabasal acantholysis and dyskeratosis.

Different genetic defects are capable of producing identical clinical anomalies. The features of our case do not prove that acantholytic dyskeratotic naevi only occur when the unknown cellular defect(s) of Darier's disease arise during embryonic development. While we believe that many cases are likely to share this pathogenesis, our patient appears to be the only reported case in whom the description 'naevoid Darier's disease' can be fully justified.

Acknowledgments

We are grateful to Dr Susanne Tiling-Grosse and Ms Eva Palfi for translations.

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Epidermolysis bullosa acquisita with oesophageal stenosis

SACHIKO MIYAGAWA, TAKASHI IIDA, HIROSHI HACHISUKA, YUKIO YAMASHINA AND TOSHIHIKO SHIRAI

Department of Dermatology, Nara Medical University, Japan
Department of Dermatology, Kurume University School of Medicine, Japan

Accepted for publication 1 April 1992

Summary

Epidermolysis bullosa acquisita (EBA) is a rare acquired subepidermal blistering disorder associated with autoimmunity to type VII collagen. Although the full clinical spectrum of EBA is still being defined, it is now known that EBA has greater clinical heterogeneity than previously suggested. We describe a patient with EBA which closely approximated the severity of the recessive form of dystrophic epidermolysis bullosa.

Epidermolysis bullosa acquisita (EBA) is an acquired mechanobullous disease characterized by autoantibodies to type VII collagen, which is localized to the anchoring fibrils within the dermo-epidermal junction.[1,2] Most reported cases of EBA demonstrate relatively mild dystrophic changes, often limited to extensor scarring, nail dystrophy, and milia. A severe variant of EBA simulating the features of the recessive type of hereditary dystrophic epidermolysis bullosa (DEB) is unusual, and has been reported only rarely.[3] We report a case of EBA with oesophageal involvement.

Case report

In April 1985, a 64-year-old man was referred to our hospital because of a severely scarring bullous disorder which had been present for 10 years. The initial lesions developed on the palms but soon spread to involve much of his body, with a pronounced acral distribution. Most blisters arose after trauma. The oral mucosa was eroded by ingestion of solid food. The patient had been seen at another hospital where a diagnosis of EBA was made (Fig. 1a). He was treated with topical steroids, but there was no improvement in his condition.

His medical history was otherwise unremarkable. There was no family history of blistering disease.

Abnormal physical findings were confined to the skin and mucous membranes. Multiple blisters and erosions were noted on the hands, feet, elbows, knees, lower part of the back, and sacral area. Scarring from previous lesions and milia was extensive. His fingers and toes showed scarring and nail dystrophy. Blisters and erosions studded the oral mucosa and the lateral aspects of the tongue. The teeth were normal for his age. Other changes included absence of body hair within the areas of scarring. The appearance of the hair on the scalp was normal.

The following laboratory investigations were negative or within normal limits: blood count, fasting blood sugar, liver function tests, serum electrolytes, serological tests for syphilis, and antinuclear antibody. The electrocardiogram and chest radiograph were also normal. Abnormal laboratory data included an elevated ESR of 53 mm/h, microscopic haematuria, and hypergammaglobulinaemia with serum levels of IgG 2779 mg/dl (normal 780–1450 mg/dl) and IgA 750 mg/dl (normal 135–340 mg/dl).

Histological and immunopathological findings

Biopsy of a fresh blister on the forearm revealed a subepidermal bulla with little inflammation. Blister formation was beneath the lamina densa of the basement membrane zone (BMZ) as demonstrated by electron microscopy (Fig. 2), and by immunohistochemical staining for type IV collagen (Fig. 3a) using a murine monoclonal antibody against human type IV collagen[4] (Miles Laboratory Inc, IL, U.S.A.) and a peroxidase–antiperoxidase staining method on frozen tissue sections.

Direct immunofluorescence microscopic studies demonstrated linear deposits of IgG and C3 at the BMZ in both the clinically normal and lesional skin. The fluorescence staining was mainly along the epidermal edge of the blister (Fig. 4a). In contrast, IgG was found on the

Correspondence: Dr Sachiko Miyagawa, Department of Dermatology, Nara Medical University, Kashihara City, Nara 634, Japan.

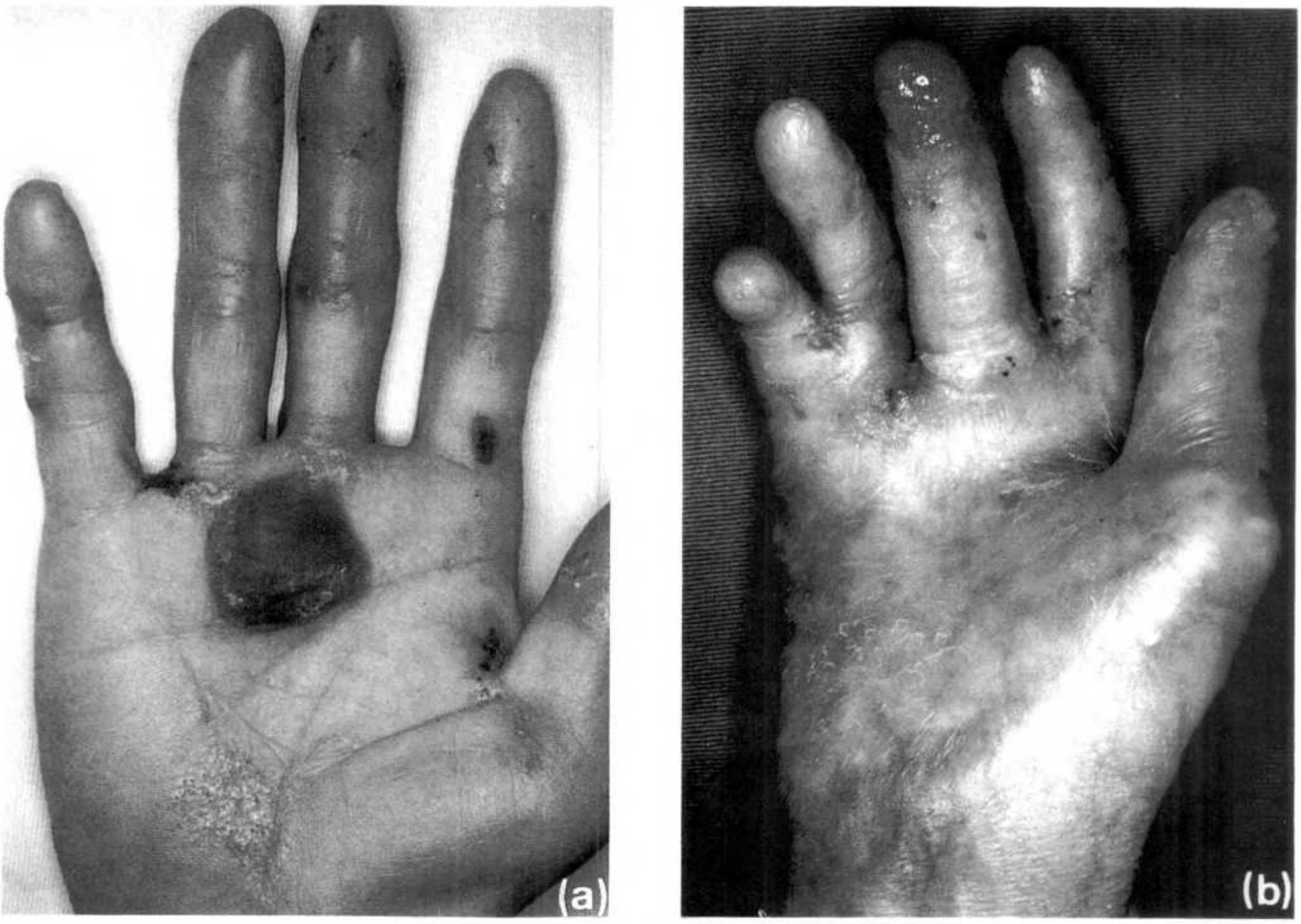

Figure 1. (a) Haemorrhagic blisters, and scarring from previous lesions on the palm in May 1977. (b) Severe cicatrization of the hand showing webbing with early pseudo-syndactyly in October 1988.

dermal side of sodium chloride-treated perilesional skin (Fig. 4b), a finding consistent with a previous study.[5]

A recent study showed that mouse monoclonal antibody against a non-collagenous domain of type VII collagen (LH 7:2, Chemicon International Inc, CA, U.S.A.) bound mainly to the roof, but also to the floor of the blister (Fig. 3b) when examined by the established method.[6]

Indirect immunofluorescence was negative for circulating anti-BMZ autoantibodies when both intact and sodium chloride-split human skin[7] were used as substrates.

Oesophageal stenosis

In addition to the cutaneous lesions, the patient had had difficulty in swallowing solid food since early 1984. A barium swallow in May 1985 showed a web-like stenosis of the upper intrathoracic oesophagus (Fig. 5). The dysphagia, and the dystrophic scarring of the hands, progressed gradually despite oral steroid therapy (a total dose of 269·5 mg betamethasone over a 10-month period). A barium swallow in July 1991, however, showed the oesophageal stenosis almost unchanged from May 1985. Oesophagoscopy revealed a circumferential diaphragm-like stricture at 24 cm from the upper teeth. The stricture prevented passage of the instrument, and dilatation was avoided owing to the extreme fragility of the oesophageal mucosa. He maintained adequate nutrition by eating slowly. His haemoglobin was 12·9 g/dl (normal 13·0–17·0 g/dl), and total protein 8·2 g/dl (normal 6·0–8·3 g/dl). The mucous membranes of the nose, conjunctiva, and trachea were not involved. The anal and genital mucosae were also unaffected.

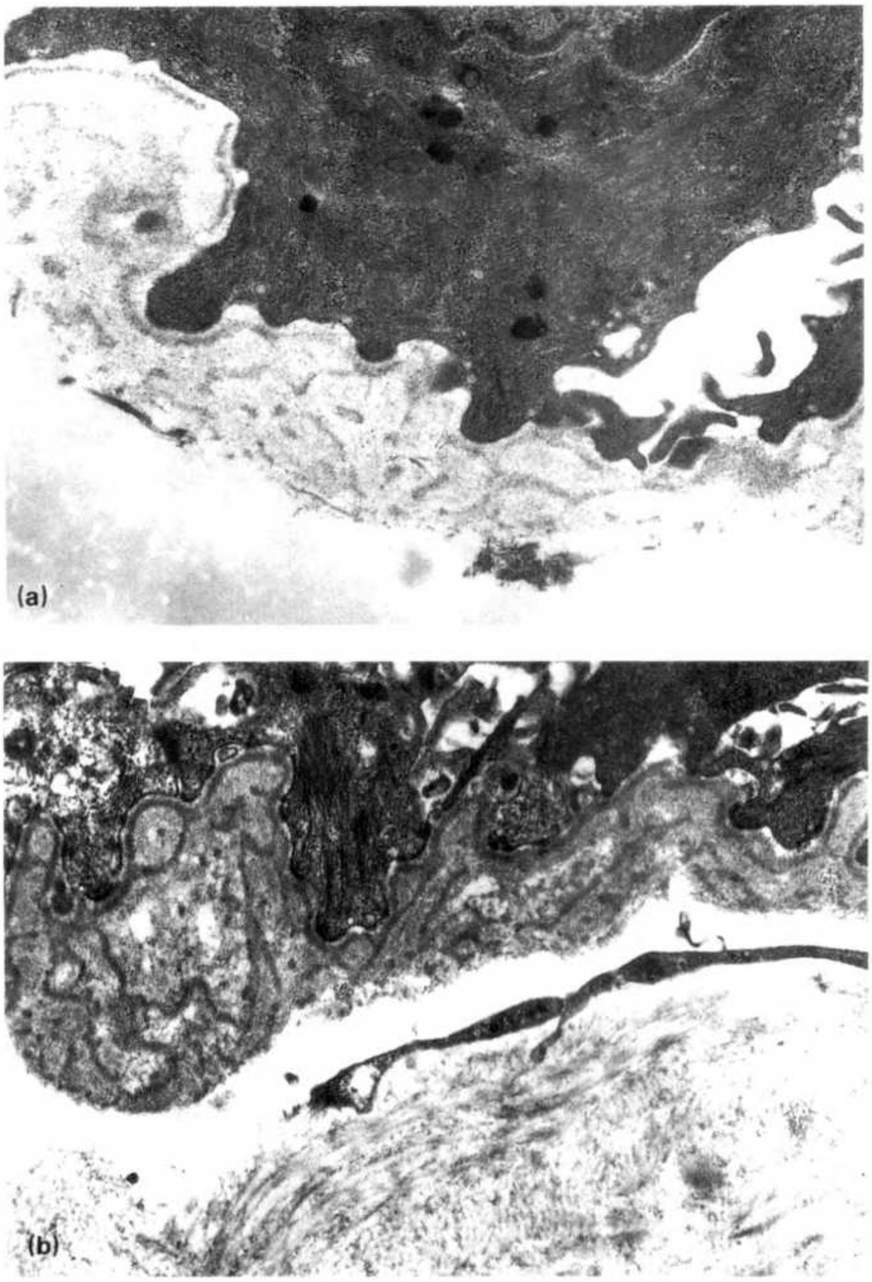

Figure 2. Electron micrograph of a blister showing sublamina densa separation. There is duplication of the lamina densa. Anchoring fibrils are indistinct. (a) roof of the blister (× 27,300); (b) perilesional skin (× 21,000).

IgA nephropathy

In June 1987, the patient was referred to the internal medicine department because of persistent microscopic haematuria and proteinuria (150–200 mg/day). A diagnosis of IgA nephropathy was made, based upon renal biopsy findings of mesangial proliferative glomerulonephritis, with IgA and C3 deposition in the mesangium. He did not have purpuric skin lesions which are associated with IgA nephropathy in some patients.[8] A biopsy of normal skin obtained from the forearm did not show vascular deposition of IgA, other immunoglobulins, C3, or fibrinogen. The patient has been receiving ticlopidine hydrochloride 300 mg/day for his IgA nephropathy. His renal function is slightly impaired.

Progress and management

The dystrophic scarring of the fingers and toes has gradually progressed (Fig. 1b). Cyclosporin, which has

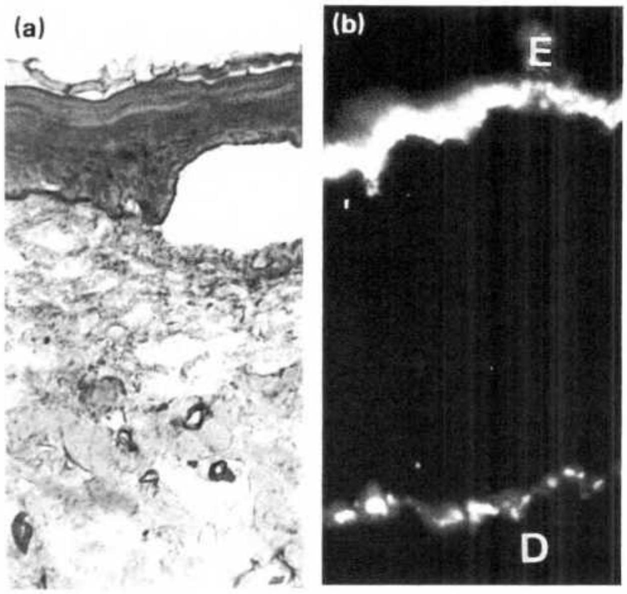

Figure 3. (a) Lesional skin immunostained for type IV collagen. Reaction products are detectable on the epidermal roof of the blister. (b) Lesional skin immunostained by monoclonal antibody against type VII collagen. Reaction products are detectable on both epidermal (E) and dermal (D) sides of the blister (× 400).

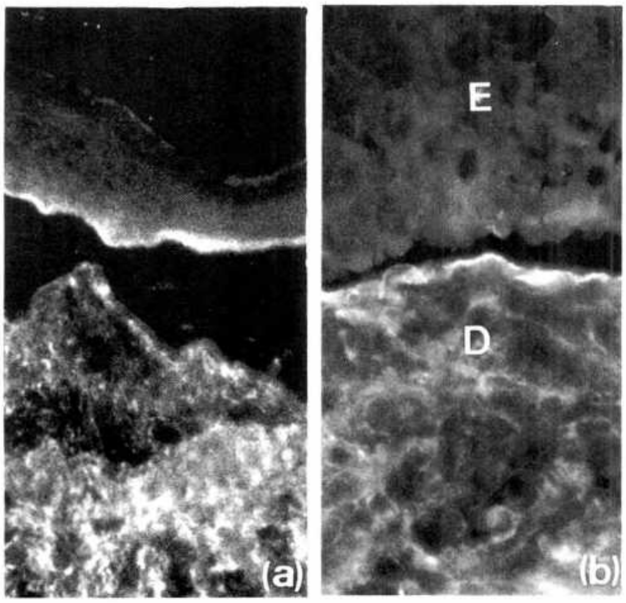

Figure 4. Direct immunofluorescence staining of the lesional skin showing (a) *in vivo*-bound IgG along the epidermal (E) edge of the blister. In contrast, (b) IgG was found on the dermal (D) side of sodium chloride-split perilesional skin.

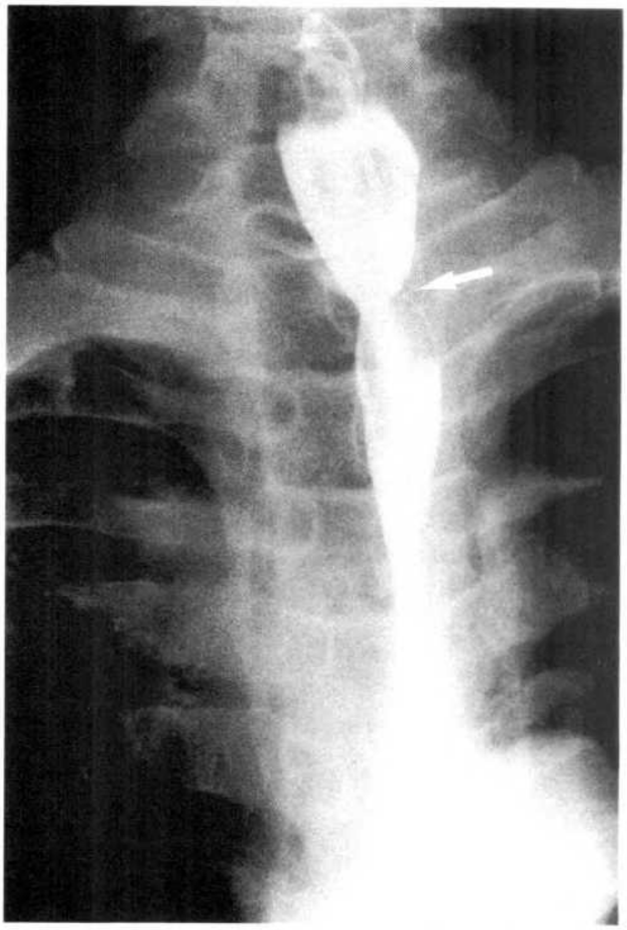

Figure 5. A radiograph of the oesophagus demonstrating a web-like stenosis (arrow) in the upper intrathoracic oesophagus.

been reported to be effective in EBA,[9] was not prescribed because of his renal disease.

Other disorders, in particular porphyria cutanea tarda, other bullous diseases and systemic sclerosis, were excluded by the history, clinical features and laboratory studies.

Discussion

Our patient's clinical features closely resembled the manifestations of recessive DEB. However, the late onset of the disease and the immunopathological findings excluded this diagnosis.

The monoclonal antibody to a non-collagenous domain of type VII collagen, which bound to both epidermal and dermal sides of the blister, showed that blister formation in our patient occurred between the dermal ends of anchoring fibrils and the lamina densa.

These observations are in agreement with published findings that type VII collagen is the major component of anchoring fibrils and the target of the autoimmune reaction in EBA.[2,10–12] Recent gene sequencing has raised the possibility that the major non-collagenous domain recognized by the EBA serum is the N-terminus of type VII collagen.[13]

EBA is known to have a wide clinical spectrum that includes a non-inflammatory mechanobullous presentation, an inflammatory vesiculobullous eruption akin to bullous pemphigoid, and a mucosal-centred disease with scarring that is reminiscent of cicatricial pemphigoid.[3,14,15] Patients with EBA often have lesions on the oral mucosa. Nevertheless, oesophageal involvement in EBA has not been well documented. In 1979 Richter and McNutt[3] reported a case of EBA who had an oesophageal stricture, and recently Stewart *et al.*[16] described a patient with EBA and coexistent oesophageal webs. Our case, together with these two reports, should broaden our perspective of EBA. We suggest that the same precautions be taken in treating patients with EBA who have oesophageal problems as are taken in patients with recessive DEB.

The association of EBA with IgA nephropathy has not previously been reported. As immunological phenomena play an important part in both EBA and IgA nephropathy, a common immunopathological link between the two diseases may exist. Further reports of new cases might help to determine whether the association is coincidental or not.

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British Journal of Dermatology (1992) **127**, 177–181.

Multiple xanthogranulomas in an adult: case report and literature review

S. ELIZABETH WHITMORE

Department of Dermatology, The Johns Hopkins Medical Institutions, 600 North Wolfe Street, Baltimore, MD 21205, U.S.A.

Accepted for publication 19 February 1992

Summary An otherwise healthy 30-year-old male developed multiple xanthogranulomas over a 1-year period. A review of the literature on adult xanthogranulomas and a tabulated outline of the differential diagnosis is presented.

Juvenile xanthogranuloma is a benign, self-healing disorder characterized by one, or commonly many, yellow, reddish–yellow, tan, or brown papules or nodules which favour the upper body. Lesions usually develop within the first few months of life, but not uncommonly may be present at birth or develop during childhood.[1,2] Xanthogranulomas in adults are thought to be relatively uncommon,[2] and when they do occur, they are most often solitary.[2,3]

A report is presented on an adult who developed multiple xanthogranulomas, and a tabulated summary is given of a literature review highlighting key features of similar entities.

Case report

A 30-year-old Caucasian male presented for evaluation of 'a dozen new moles'. He stated that initially the 'moles' appeared on the back, but subsequently developed on the abdomen and arms. The lesions were asymptomatic.

His past medical history included childhood-onset allergic asthma, nasal polyps, chronic sinusitis, and recently detected mild hypercholesterolaemia. For the latter condition, he had attempted to reduce his dietary

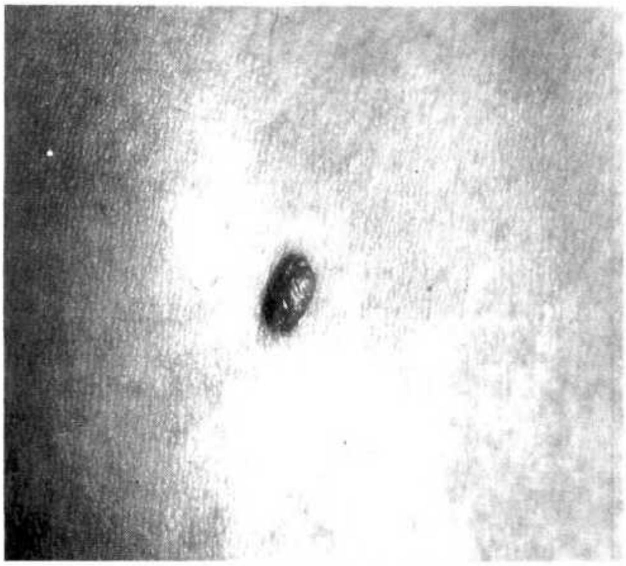

Figure 1. One of 10 xanthogranulomas scattered over the trunk and arm.

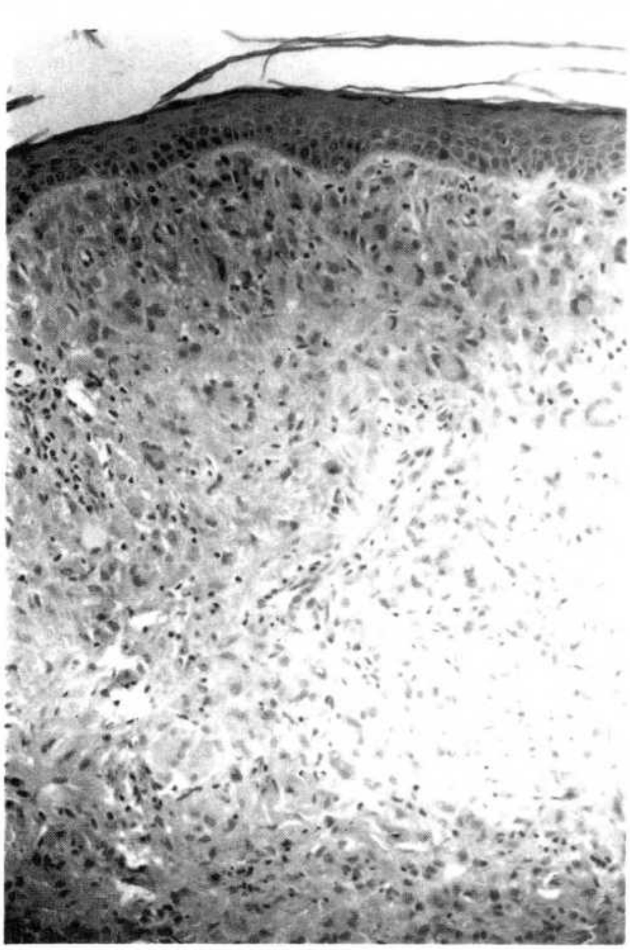

Figure 2. Photomicrograph showing a nodular dermal aggregate of histiocytic cells and scattered Touton giant cells (haematoxylin and eosin × 125).

Table 1. Adult xanthogranuloma: differential diagnosis

		Clinical				Histopathology		
	Ages affected	Lesion number/ distribution	Lesion colour and morphology	Oral mucosal involvement	Additional sites of involvement and systemic associations	Characteristic circumscribed dermal infiltrate of	Langerhans granules	Natural history
Adult xanthogranuloma[3,12,13]	24–80[3]	Usually solitary, but up to 26/ scattered	Yellow, orange, or tan papulonodules	(—)	(—)	Foam cells, foreign body and Touton giant cells, histiocytes, lymphocytes, eosinophils	(—)	Nine cases, six solitary lesions excised/ three multiple lesions no resolution on follow-up <3 years[3]/usually involute spontaneously, but may be present longer than juvenile xanthogranuloma, even up to 15 years[12]
Xanthoma disseminatum[13–18]	Children and adults[15]	Up to 100's/face trunk, extremities; coalesce in flexural areas	Initially red-brown, become yellow papules	Common	Conjunctival, corneal, pharyngeal, laryngeal, tracheal, and bronchial infiltration/nodules possible (may compromise respiratory function), presumed pituitary involvement causing diabetes insipidus in 50%, other organ involvement rarely[15,16], one case each of associated Bence-Jones proteinuria[14], Waldenstrom's macroglobulinaemia[17] and multiple myeloma[18]	Histiocytes, foam cells, inflammatory cells, numerous Touton giant cells ('indistinguishable from juvenile xanthogranuloma'[19])	(—)	Usually resolution after several years; rare progressive cases reported[15,16]

Papular xanthoma[7,12,20,21]	Children and adults	Multiple/face, trunk, or generalized	Yellowish papulonodules (2–15 mm)	Possible	(—)	Histiocytes, foam cells, Touton giant cells	(—)	Usually resolution after several years; rare progressive cases reported[7]
Generalized eruptive histiocytomata[12,22]	Usually adults, children possible	Many to 1000's/ widespread especially face, trunk and proximal extremities	Flesh-coloured to bluish, brownish-red papules (3–10 mm)	Rare	(—)	Monomorphous histiocytic infiltrate	(—)	Usually complete or incomplete (brown macules) resolution after a few years[12,22]
Histiocytosis X (HX) with papular xanthoma-like lesions (three reports)[23–25]	7-year-old[23]	Several/ trunk[23]	Yellow–brown papules	(—)	Diabetes insipidus, lytic bone lesions, mental retardation[23]	Xanthoma cells, histiocytes, some multinucleated giant cells and an inflammatory infiltrate[23]	Not reported[23]	Not given[23]
	25-year-old[24]	Many/ generalized[24]	Yellow–brown papulo-nodules (3–10 mm)[24]	(—)	None[24]	Foam cells major cell type[24]	Present in some non-xanthomatous histiocytes[24]	No resolution 1.5 years after onset[24]
	Five children, 1 adult[25]	1–10 lesions/ randomly distributed[25]	Sulphur-yellow orange, rose, red–brown papulo-nodules[25]	(—)	Five patients with disseminated HX, 1 with cutaneous HX only (clinically xanthomatous lesions developed near the end or after [up to 48 months] treatment of HX with vinblastine in the five patients so treated)[25]	Large foamy, occasionally multinucleated cells, no Touton cells[25]	Present in the original HX lesions, absent in the subsequently developing xanthomatous lesions[25]	Resolution in 2–48 months[25]
Eruptive xanthomas[26]	Children and adults	Many/ appear in crops/ extensor thighs and buttocks	Yellow with red halo papules	(—)	Lesions are caused by elevated serum triglyceride levels (usually >1000 mg/ml) as may be seen in some hyperlipidaemias	Foam cells +/− lymphocytes, histiocytes, and neutrophils	(—)	Dependent on elevated triglyceride (TG) level; a significant TG reduction leads to lesion involution

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* Involvement of the cerebrum, cerebellum, liver, gallbladder, pancreas, kidney, uterus, muscles, and lymph nodes has been reported.

cholesterol intake. He denied polydypsia and polyuria. There was a family history of mild hypercholesterolaemia.

Examination revealed 10 3–5-mm-diameter yellowish–brown, dome-shaped papules scattered over the back, abdomen, and flexor aspect of the left arm (Fig. 1). The eyes and oral mucosa were normal. An excision biopsy of one of the lesions was performed. Histology revealed a circumscribed mixed dermal infiltrate of Touton giant cells, foam cells, and histiocytic and lymphoid cells (Fig. 2). S-100 protein stain showed staining of epidermal melanocytes and scattered dermal interdigitating reticulum cells, but no staining of the cells of the infiltrate, indicating that the lesional histiocytic cells were not Langerhans cells.

Laboratory studies, including a full blood count, biochemistry, and lipid profile (cholesterol, HDL, LDL, and triglycerides), were all within normal limits. Ophthalmological examination was normal.

Discussion

Naevoxanthoendothelioma was initially fully described in 1912 by McDonagh.[4] In 1954, Helwig and Hackney[1] renamed the condition juvenile xanthogranuloma, pointing out that it occurred in infants and children, was composed of lipid-containing histiocytes, and was not a naevus. Nine years later, Gartmann and Tritsch[5] first reported xanthogranuloma in an adult. In 1976, Rodriguez and Ackerman[3] reported nine cases of xanthogranulomas in adults seen at their institution over a 15-month period. Six patients had solitary lesions, while three each had two, three, and 26 lesions, respectively.

Gianotti and Zina[6] were the first to recognize and classify juvenile xanthogranulomas clinically into micronodular and macronodular forms. In the micronodular type, several to hundreds of 2–5 mm, red to yellow, firm, dome-shaped papules are scattered predominantly over the upper body. The macronodular type is similar, except lesions are less numerous and are 1–2 cm in size.[2,6,7] With all cutaneous juvenile xanthogranulomas, volar and mucous membrane lesions are uncommon.[2] Visceral involvement with juvenile xanthogranuloma most often affects various parts of the eye and may cause significant morbidity. Most serious are uveal lesions which may lead to glaucoma, recurrent hyphaema (anterior chamber haemorrhage), miosis, photophobia, or iris discoloration.[8,9] In the macronodular type, other visceral lesions may occur in the colon, testes, ovaries, lungs, pericardium, and bones. These lesions are usually inconsequential, but involvement of the three latter sites has led to dyspnoea,[10] cardiovascular compromise and respiratory distress (due to haemopericardium),[11] and pathological fracture.[2] To date, there are no reports of adults with cutaneous xanthogranulomas having visceral lesions, although adults (and children) may have isolated ocular xanthogranulomas.[1,9,12]

The histopathological features of xanthogranulomas are dependent on their stage of evolution. Early lesions consist of a monomorphous histiocytic infiltrate in the upper dermis. Mature lesions show lipid-laden histiocytes ('foam cells'), infiltration by eosinophils, plasma cells, and neutrophils, and foreign-body and Touton giant-cell formation. More advanced lesions may show fibrosis. Stains for fat are positive, while S-100 staining to identify Langerhans cells is negative.[12,13]

The differential diagnosis of multiple xanthogranulomas in an adult includes xanthoma disseminatum, papular xanthoma, generalized eruptive histiocytomata, histiocytosis X with xanthoma-like lesions, and eruptive xanthomas. Key features of these entities are outlined in Table 1.

Acknowledgment

The author would like to thank Dr Thomas Horn for providing the histopathology slides.

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British Journal of Dermatology (1992) **127**, 182–184.

Cutaneous granulocytic sarcoma (chloroma) presenting as the first sign of relapse following autologous bone marrow transplantation for acute myeloid leukaemia

D.W.S.HARRIS, L.S.OSTLERE AND M.H.A.RUSTIN

Department of Dermatology, The Royal Free Hospital and School of Medicine, Pond Street, London NW3 2QG, U.K.

Accepted for publication 28 February 1992

Summary A 25-year-old man suffering from acute myeloid leukaemia developed a solitary lesion on the upper abdominal wall 6 months after receiving an autologous bone marrow transplant. The lesion was a chloroma and proved to be the only evidence of clinical relapse. This is the first reported case of this rare condition occurring following bone marrow transplantation.

Cutaneous chloromas (granulocytic sarcomas) are rare tumours composed of immature cells of the granulocyte series. Clinically they appear as green-coloured dermal nodules. The green tinge is due to the presence of granulocyte peroxidase. The colour can be enhanced by wetting the lesion with an alcohol swab. Chloromas are most often seen in young patients suffering from acute myeloid leukaemia (AML). The appearance of such cutaneous leukaemic infiltrates is generally a poor prognostic sign—nearly 90% of patients die within 1 year of diagnosis.[1] The prognosis is worst in chronic myeloid and acute myeloid leukaemias.[2] The sites of predilection are bone and periosteum—in particular the cranium, orbit, maxillae, sternum and vertebrae. The next most common sites are soft tissues, of the chest wall and arms, the skin, kidneys, gut and lymph nodes. Localization of such deposits results in focal disturbances—spinal cord compression,[2] facial nerve paresis, exophthalmos,[2] obstructive nephropathy and right ventricular failure[3] have all been recorded. We present a case of solitary cutaneous chloroma heralding systemic relapse 6 months after a successful autologous bone marrow transplant.

Case report

A 25-year-old man developed a hard, asymptomatic, green nodule on the upper abdomen (Fig. 1) 6 months after receiving an autologous bone marrow transplant for acute myeloid leukaemia. He was in good general health, with no specific complaints, and physical examination was otherwise normal. His full blood count on presentation showed a haemoglobin of 11·6 g/dl, white cell count $5{\cdot}5 \times 10^9$/l and platelets 180×10^9/l. Bone marrow examination and a lumbar puncture to detect possible CNS relapse were both normal.

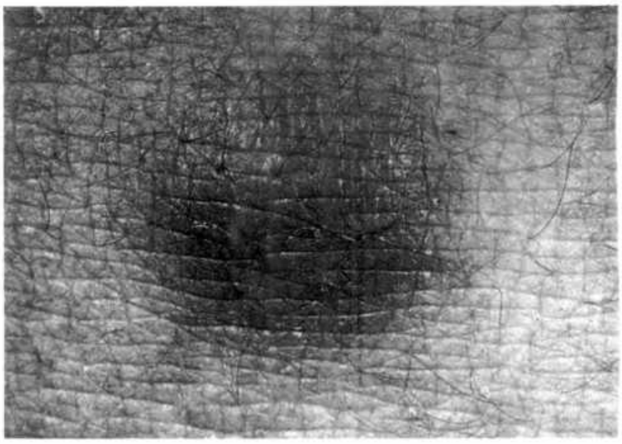

Figure 1. Hard dermal nodule on right upper abdominal wall.

The diagnosis of acute myeloid leukaemia had been made a year prior to presentation. At that time he complained of fever, malaise, tiredness, bone pain and gum bleeding. A full blood count showed a haemoglobin of 7·8 g/dl, white cell count $1{\cdot}7 \times 10^9$/l with 2% blasts, and platelets 47×10^9/l. Initial treatment consisted of two courses of chemotherapy comprising adriamycin, cytosine arabinoside, and 6-mercaptopurine. His disease remitted and in July 1990 he underwent an autologous bone marrow transplant. His post-transplant course was complicated by severe gingival ulceration, febrile episodes, and an interstitial pneumonitis with bilateral

Correspondence: Dr D.W.S.Harris, Department of Dermatology, The Royal Free Hospital, Pond Street, London NW3 2QG, U.K.

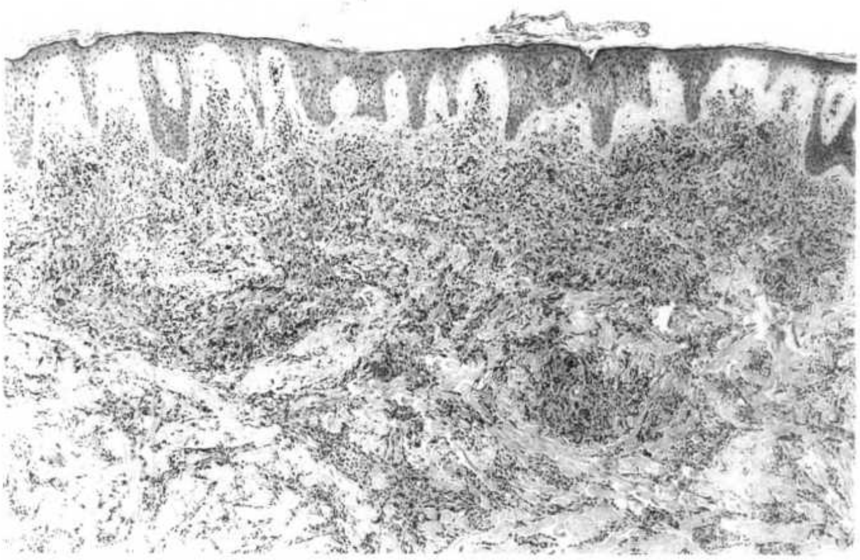

Figure 2. Biopsy of lesion showing a heavy infiltrate of cells within the dermis and extending into the subcutis (haematoxylin and eosin × 10).

pleural effusions. An atypical mycobacterium was subsequently cultured from blood and bone marrow and identified as *Mycobacterium chelonei*. He was started on anti-tuberculous therapy and his fever and respiratory symptoms resolved.

A skin biopsy of the lesion on his upper abdomen showed an infiltrate of cells with pleomorphic vesicular nuclei, conspicuous nucleoli and granular acidophilic cytoplasm, between the dermal collagen bundles (Figs 2 and 3). The cytoplasmic granules in these cells stained positive for chloroacetate esterase, confirming their identity as granulocytic cells. The infiltrate extended into the subcutis and was well demarcated from uninvolved skin laterally. A diagnosis of granulocytic sarcoma (chloroma) was made.

The nodule was completely excised and the site treated with a 5-day course of local radiotherapy. Following this, he was given a further course of chemotherapy consisting of daunorubicin, cytosine arabinoside and thioguanine. He remained well for the following 3 months, but thereafter the leukaemia relapsed, he deteriorated rapidly, and died.

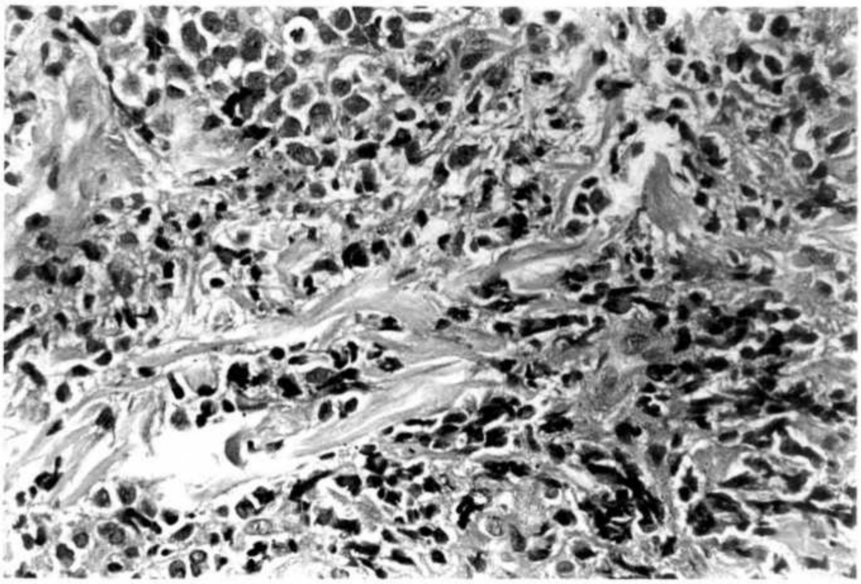

Figure 3. The infiltrate is composed of myeloid precursors with prominent vesicular nuclei, conspicuous nucleoli and cytoplasmic granules. It can be seen extending between the collagen bundles (haematoxylin and eosin × 400).

Discussion

Granulocytic sarcomas are rare leukaemic tumours that occur in myelodysplastic syndromes as well as in leukaemic states. They receive no mention in a major British textbook of dermatology.[4] Their incidence in a series of 478 patients with myelogenous leukaemia was 3·1%, and was twice as high in patients with chronic compared with acute granulocytic leukaemia.[5] The pathogenesis of granulocytic sarcomas is uncertain, although it is speculated that trauma-induced extravasation of myelodysplastic cells leads to their local replication within the skin.[6] The tumour was first described by Burns in 1811[7] and its association with leukaemia was established by Dock in 1893.[8] King,[9] in 1853, named the tumour 'chloroma' but the preferred designation of granulocytic sarcoma was coined in 1966 by Rappaport.[10]

Although it is usual for granulocytic sarcomas to follow the development of leukaemia, several cases have been reported in which their occurrence antedated the diagnosis of leukaemia.[11-13] In such cases virtually all patients develop leukaemia within a mean of 10 months.[11 14]

There are very few case reports of cutaneous granulocytic sarcoma.[11,13,15,16] Single lesions are the most common presentation.[11,17] Chloracetate esterase and antilysosomal immunoperoxidase stains are equally reliable in confirming the granulocytic nature of the tumour cells.[11] There is no established therapy for the condition although electron beam, conventional radiotherapy and chemotherapy have been tried. These palliative measures produce prompt shrinkage of the lesions but relapse subsequently occurs. In a review of 16 patients seen over 25 years, seven patients did not develop leukaemia, the longest follow-up being 16 years. All patients were given systemic chemotherapy.[18]

Acknowledgment

We would like to thank Dr H.G.Prentice, Director, Bone Marrow Transplant Unit, RFH, for allowing us to report his patient.

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Correspondence

Correlation between the pretreatment number of blisters and the time to control bullous pemphigoid with prednisone 1 mg/kg/day

Sir, Systemic steroids constitute the principal treatment of bullous pemphigoid (BP).[1] Adverse effects of steroids, which are related to dose and duration of therapy, contribute to mortality in BP patients.[2] The age at onset of the disease is an important prognostic factor in BP,[3] and it has been suggested that the extent of disease might also be a prognostic factor.[4] In a previous pharmacokinetic study, we suspected a link between the pretreatment number of blisters and the time required for their resolution,[5] and the aim of this present study was to confirm a correlation between the pretreatment number of blisters and the time to control BP with prednisone 1 mg/kg/day.

We retrospectively reviewed 145 patients who were admitted to hospital for treatment of BP between 1982 and 1991. Patients were included in the study if they fulfilled the following criteria. (i) Complete clinical, biological and histological data were available. (ii) There were typical features of BP as previously defined.[1] BP was differentiated from other subepidermal bullous diseases (i.e. epidermolysis bullosa acquisita or benign mucosal pemphigoid) on the grounds of clinical features, histology and outcome.[6] (iii) Patients had been treated with oral prednisone 1 mg/kg/day (Cortancyl®, Laboratoire Roussel) alone. This minimized the risk of phamacokinetic resistance because prednisolone is the active biological metabolite, and pharmacokinetic data in healthy volunteers has shown that oral prednisone produces a better systemic prednisolone bioavailability than oral prednisolone.[7] (iv) No patient had any concomitant treatment which could be of benefit in BP, or be responsible for a drug interaction;[2,8,9] Patients whose previous treatment (for example, with dapsone) had been ineffective, were included in the study only if the number of blisters remained unchanged until the onset of corticosteroid therapy. The number of new blisters was counted 2 days (day_{-2}) and 1 day (day_{-1}) before treatment, and every day after commencement of treatment (day_1, day_2, day_3, . . ., day_n). The number of blisters on the first day of corticosteroid therapy (day_0) was calculated as the mean of the number of blisters on day_{-2} and day_{-1}. The day of control of the disease (day_x) was defined as the first day when no new blisters developed. The correlation was tested using a linear regression (StatWorks Software, U.S.A.). Twenty-three patients fulfilled these criteria and were included in the study [16 female, seven male; mean age 79·9 years (68–99)]. Eight patients (35%) received various ineffective treatments prior to therapy with prednisone 1 mg/kg/day: dapsone ($n=4$), low-dose systemic corticosteroids ($n=3$), intravenous immunoglobulins ($n=1$). The mean number or blisters on day_0 was 54 (2–325). The mean day of BP control (day_x) was 5·7 days (2–15). We found a significant correlation between the pretreatment number of blisters and the time to control BP with oral prednisone 1 mg/kg/day ($r=0{\cdot}65$; $P<0{\cdot}001$) (Fig. 1): for example, the duration of treatment to achieve suppression of blister formation was 4 days for a patient presenting with 34 blisters, whereas it was 15 days for another patient presenting with 125 blisters.

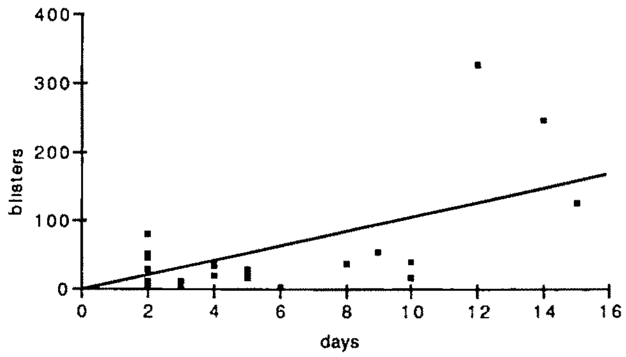

Figure 1. Significant correlation between the pretreatment number of blisters and the time to control BP with oral prednisone 1 mg/kg/day ($r=0{\cdot}65$; $P<0{\cdot}001$).

We have been able to confirm a correlation between the number of pretreatment blisters and the time to control the disease with prednisone 1 mg/kg/day in our BP population. Indeed, the pretreatment number of skin blisters may influence the duration of corticosteroid therapy in BP. Patients with a high initial number of blisters should not necessarily be considered as non-responders to prednisone 1 mg/kg/day if their disease is not rapidly controlled. Control may be obtained by simply prolonging the duration of treatment with this dose of prednisone rather than changing the dose of steroid or adding other treatment (i.e. immunosuppressive agents or plasma exchange). In contrast, patients presenting with a low number of blisters could benefit from a shorter duration of treatment with the initial corticosteroid dose.

BP patients are usually elderly and are vulnerable to adverse effects of drugs, which may have serious consequences.[3] Our study suggests that the initial number of blisters in BP may provide a guide to the duration of treatment with prednisone 1 mg/kg/day required to control the disease. Further studies are required to evaluate this guide in the management of BP patients, with particular reference to influences on the incidence of adverse effects and on mortality.

*Services de Dermatologie et de *Pharmacologie,*
*Hôpital Henri Mondor et *Groupe Hospitalier,*
Pitié-Salpêtrière,
94010 Créteil et 75013 Paris,
France

O.Chosidow†
V.Saada
B.Diquet*
J.C.Roujeau
J.Revuz

† *Present address: Unité de Dermatologie,*
Groupe Hospitalier Pitié-Salpêtrière,
75013 Paris,
France

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Exacerbation of bleeding tendency in a patient with haemophilia A, during treatment with isotretinoin

Sir, We report the case of a patient suffering from haemophilia A who developed an increased frequency of bleeds, and an overall increase in requirement for factor VIII, during treatment of his acne with isotretinoin (Roaccutane®).

A 17-year-old boy with haemophilia A presented with a 5-year history of acne. He had scarring papulo-pustular acne of grade 2·5 on the face and 2·0 on the back and shoulders, using the Leeds grading technique. His previous therapy included oxytetracycline 500 mg b.d. for 1 year. We subsequently supervised therapy with doxycycline 100 mg daily for 4 months and erythromycin 1 g daily for 8 months, both used in conjunction with topical 5% benzoyl peroxide. An initial improvement was seen with each regime, but his acne did not clear.

Treatment with isotretinoin was started in January 1991, and the dose was gradually increased over 7 weeks to a final maintained dose of 1 mg/kg/day. Treatment was discontinued after 4 months. One month later there was minimal activity of the acne on the back and no new facial lesions. The associated improvement in the patient's self-confidence was striking as his previous character had been rather depressive and introverted.

His haemophilia A was classed as moderately severe, with a factor VIII level of 3·5%. He experienced episodic spontaneous bleeding into muscles and joints, and excessive bleeding after minor trauma. These episodes were managed with self-administered factor VIII. During treatment with isotretinoin there was an increased frequency of bleeds and hence an increased requirement for factor VIII (Table 1). There was also an alteration in the character of his bleeds, which were usually evenly distributed between joint and soft tissue sites. Whilst taking isotretinoin nine out of ten bleeds were within soft tissues. Several minor epistaxes occurred during isotretinoin therapy. These were controlled by simple measures and required no additional factor VIII. Haemoglobin concentration, platelet count, and liver function tests showed no significant change during treatment.

Table 1. Number of bleeds and factor VIII requirement. Four-monthly intervals before, during, and after treatment with isotretinoin

Date	No. of bleeds	Units of factor VIII required
Jan –Apr. 1990	5	14,700
May–Aug. 1990	5	8100
Sep.–Dec. 1990	4	3600
*Jan.–Apr 1991	10	18,300*
May–Aug. 1991	5	6300

* Period of treatment with isotretinoin.

Side-effects of isotretinoin are well documented,[1] particularly cutaneous manifestations, teratogenic potential, and hyperlipidaemia. Haematological abnormalities are either rare or have not given cause for concern in normal individuals. There are two published reports of thrombocytopenia following isotretinoin therapy for acne.[2,3] Manufacturers have details of a further 19 cases on file. In the published cases, thrombocytopenia was reversed within days of withdrawing isotretinoin therapy. In one case[2] rechallenge initiated a further episode of thrombocytopenia, and antiplatelet antibodies were demonstrated. Thrombocytopenia did not occur in our patient.

Retinoids have been shown to stimulate the synthesis and release of tissue-type plasminogen activator by cultured human synovial fibroblasts,[4] suggesting a potential for increased fibrinolytic activity *in vivo*. Studies in normal human volunteers, following a single oral dose of etretinate, failed to show any influence on the fibrinolytic system.[5] We are unaware of any reports of the effect of isotretinoin or related retinoids on the fibrinolytic system during prolonged administration.

On direct questioning, the patient did not report any alteration in the character of his bleeds whilst receiving treatment with isotretinoin. Review of his records, however, revealed an increased frequency of bleeds and an overall increase in the requirements for factor VIII. We also noted that nine out of ten bleeds were within soft tissues, rather than the more usual distribution between soft tissues and joints. The patient was aware that myalgia may occur during treatment and no strenuous or unaccustomed physical exercise was taken during the time he was treated with isotretinoin. His soft tissue bleeds did not have any unusual characteristics and myalgia was not felt to be contributing to his symptoms at these soft tissue sites.

The increased bleeding tendency seen in this case suggests that isotretinoin was influencing haemostasis. From previously published work, [4] we suggest that isotretinoin may have stimulated plasminogen activator activity and hence fibrinolysis. Whilst this would be clinically insignificant in normal individuals treated with isotretinoin, it became manifest within the setting of haemophilia A. Further detailed studies are in progress.

The improvement in affect and self-confidence seen in this teenager was striking. It is well documented that acne impairs self-confidence[6] and employment opportunities.[7] These features are also recognized in haemophiliacs. Adequate treatment of acne is therefore particularly desirable in these patients. We would, however, recommend caution when using isotretinoin in patients with haemophilia until further detailed studies of fibrinolysis *in vivo* are available.

Departments of Dermatology G.M.DOOTSON
*and *Haematology,* J.KEIDAN*
Queen Elizabeth Hospital, J.A.R.ANDERSON
King's Lynn, Norfolk PE30 4ET, UK

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Generalized pustular psoriasis associated with bacterial endocarditis of the anterior papillary muscle

SIR, Generalized pustular psoriasis (GPP) is an uncommon condition first described by von Zumbusch in 1910.[1] Exogenous precipitating factors are well recognized and include drugs, in particular a reduction in the dose of systemic or potent topical steroids,[2] pregnancy,[3] sunburn,[4] and infections.[5] We report a case of GPP and bacterial endocarditis, an association which, to our knowledge, has not been described previously. This patient is of particular interest as the endocarditis affected the papillary muscle, a recognized but unusual phenomenon.

A 24-year-old Moroccan man presented with a 1-week history of a rash affecting the dorsa of his hands and feet. He gave a 20-year history of psoriasis affecting his scalp, arms and groins. He had used about 50 g of betamethasone dipropionate 0·05% (Diprosone®) cream per week on the affected areas for many years, and 4 days before admission this had been changed to betamethasone valerate 0·1% cream in a 1 in 4 dilution (Betnovate RD®) which he had used sparingly. There was no history of rheumatic fever.

On examination he was apyrexial. There were erythematous plaques, some of which had a peripheral scale, on the dorsa of his hands, forearms, and feet. Scattered pustules were seen within the plaques. There was thick scale on the scalp. General examination was otherwise normal. Over the next few days the plaques coalesced and the erythema and pustulation became generalized. He was febrile, his temperature spiking up to 39°C.

On admission the full blood count, biochemistry, C-reactive protein and urine microscopy were normal. Subsequently the white cell count rose to 29×10^9/l with a neutrophilia, serum albumin fell from 45 to 29 g/dl, the ESR increased to 44 mm/h and the C-reactive protein level to 109 mg/l (<10). The ASO titre was <200 U/ml. HIV antibody was not detected. A throat swab grew normal upper respiratory tract flora. Initially cultures of swabs from the pustules were sterile, but repeat swabs from the arm and face 2 weeks after admission produced a heavy growth of *Staphylococcus aureus*. Ten sets of blood cultures were taken and two grew coagulase positive *Staphylococcus aureus*. An electrocardiogram and chest radiograph were normal. A skin biopsy taken from the dorsum of the hand was consistent with the diagnosis of pustular psoriasis and the biopsy site healed well, with no evidence of secondary infection.

Treatment with emollients and a weak topical steroid was started and after 3 days etretinate 75 mg/day was added. The dose of etretinate was gradually reduced to 35 mg/day because of side-effects. However, there was no improvement in his condition, and he remained erythrodermic, with recurrent formation of sheets of pustules and widespread desquamation.

An echocardiogram was performed because of persistent fever, and showed a long sessile echogenic structure of at least 10 mm length, consistent with a vegetation. This was attached to what appeared to be a redundant false chordae tendineae of the anterior papillary muscle in the left ventricle.

In view of the risk of sepsis with an indwelling intravenous catheter, he was treated with single intravenous doses of flucloxacillin and gentamicin, and oral flucloxacillin and fucidin. Within a week of starting antibiotic treatment he became apyrexial. After 3 weeks of treatment a repeat echocardiogram showed the echogenic structure was much reduced in size, and 3 weeks after completing a 6-week course of antibiotics it cleared.

The erythroderma and pustulation gradually began to improve 3 weeks after admission, at about the time that antibiotics were started, and after 6 weeks he had residual mild erythema only. The dose of etretinate was gradually reduced, and stopped after 8 months. His psoriasis remained in remission without treatment a year later.

The withdrawal of potent topical steroids is a well-recognized precipitant of GPP.[2,6] Our patient had used 50 g/week of a potent topical steroid on his psoriasis for many years, and this had been replaced shortly before admission by a moderately potent preparation which he had been advised to use sparingly. This reduction in topical steroid potency may have played a part in the precipitation of GPP. Infections are another well-recognized precipitant of GPP. Fourteen of the 104 cases of GPP reported by Baker and Ryan appeared to have been provoked by infection.[2] Viral or streptococcal upper respiratory tract infection[2,3,4] and β-haemolytic streptococcal infection of the skin[3] have been implicated. It is possible that the endocarditis was a contributing factor in the development of GPP in our patient. *Staphylococcus aureus* causes approximately 20% of all cases of infective endocarditis and in about half of these a predisposing heart lesion is not recognized, and the portal of entry may be a localized abscess or furuncle.[7] Anomalous chordae tendineae of the left ventricle have been reported in 2·2–34% of patients examined by echocardiography.[8,9,10] Although anomalous tendons represent a fairly common and usually benign phenomenon,[8] they are only occasionally involved in endocarditis. It is probable that our patient developed staphylococcal endocarditis on an abnormal chordae tendineae, and secondary infection of the skin with *S. aureus* was the most likely source of infection. Colonization of diseased skin by *S. aureus* is very common, particularly when the integrity of the epidermal barrier is disrupted.[11] It is therefore perhaps surprising that staphylococcal endocarditis is not seen more often, considering the frequency of anomalous left ventricular chordae tendineae.

Department of Dermatology
*and *Academic Unit of*
Cardiovascular Medicine,
Westminster Hospital,
Horseferry Road,
London SW1, U.K.

L.S.OSTLERE
F.AKHRAS*
J.A.A.LANGTRY
R.C.D.STAUGHTON

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Facial oedema and acne vulgaris

SIR, Fixed facial oedema can occur as a rare complication of acne vulgaris. It has been successfully treated with oral steroids[1,2] and with isotretinoin,[3,4] but antibiotics are said to be ineffective.[1,5] We observed a patient with marked facial oedema which slowly subsided after clearance of acne.

A 20-year-old man presented with a 2-year history of upper facial swelling. The swelling had initially been variable and minor but had deteriorated and became fixed over the previous 3 months. Examination showed non-pitting oedema of the forehead, eyelids and upper cheeks associated with slight erythema. He also had mild facial acne with comedones and pustules most evident on the forehead and chin (Fig. 1).

Biopsy showed dermal oedema, follicular plugging and a chronic inflammatory infiltrate. Direct immunofluorescence and a periodic acid Schiff stain were negative. X-rays of chest, skull and sinuses were normal. Routine biochemistry and haematology screens revealed no abnormality. Serum creatine phosphokinase, immunoglobulins, serum complement and an antibody screen were all within normal limits.

A diagnosis of fixed facial oedema complicating acne vulgaris was made. Negative clinical and laboratory findings

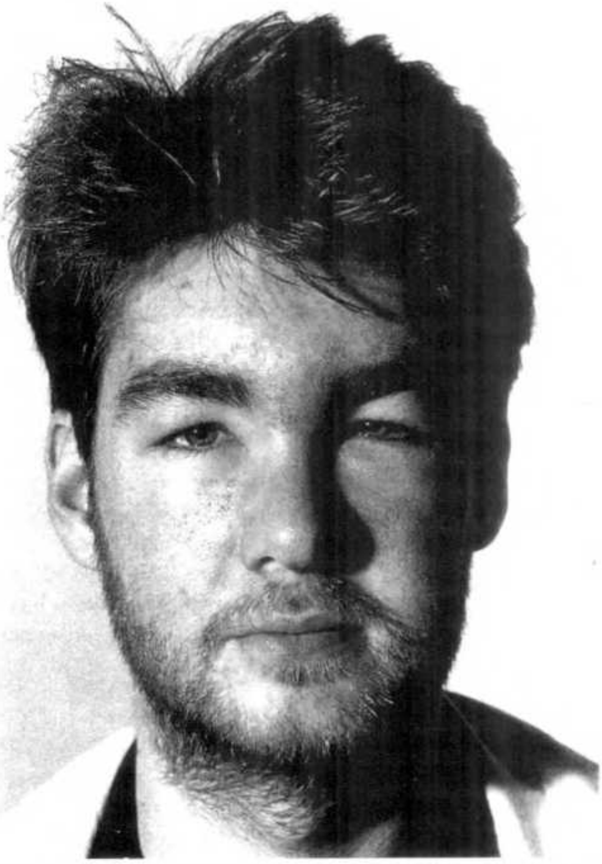

Figure 1. Mild acne vulgaris and disfiguring oedema of the upper face.

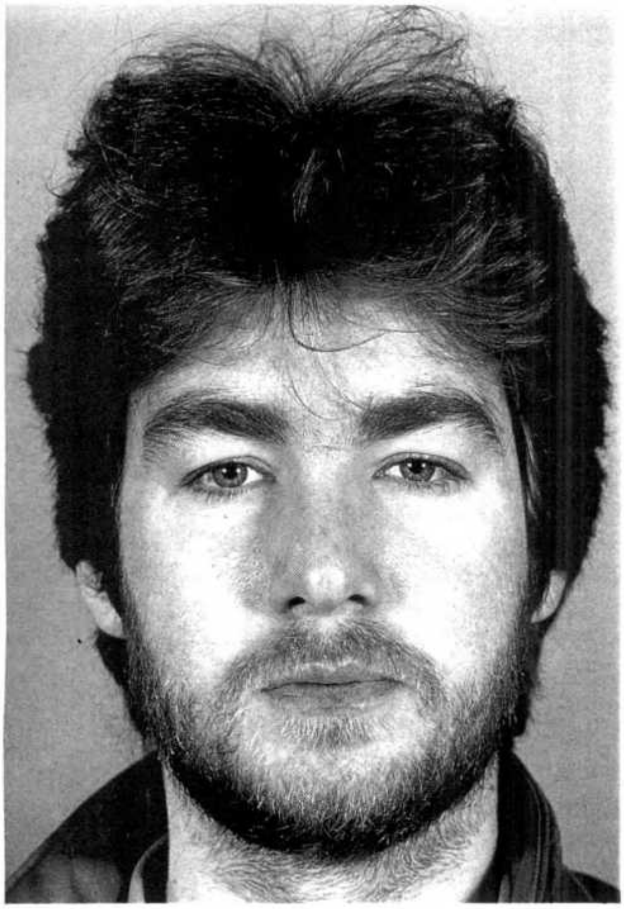

Figure 2. Marked improvement of swelling 13 months after resolution of acne.

excluded cellulitis, rosacea, vascular obstruction, angioedema, dermatomyositis, granulomatous infiltration, and Melkersson–Rosenthal syndrome.

The patient was treated with oxytetracycline for 4 months. There was resolution of the acne but the oedema persisted unchanged. A 1-week course of oral prednisolone had no effect. Treatment with an oral diuretic was also ineffective. Isotretinoin was commenced but was stopped within 4 weeks because of intolerance. Antihistamines produced no benefit. Four months later and some 6 months after resolution of the acne the facial oedema began to improve. Over the following 7 months the swelling gradually subsided to leave only a residual coarsening of the features (Fig. 2). The head circumference decreased by 1·6 cm from its measurement at the height of the swelling.

The patient made a satisfactory recovery from what was a disfiguring deformity. Because of the variety of treatments used and the delay before response it is difficult to assign credit for the improvement to any particular treatment. It is possible that the successful treatment of the acne with an oral antibiotic contributed to the resolution of the swelling. As acne-induced inflammation is thought to be the initial cause of oedema[5] such treatment has obvious potential benefit. The delay before improvement occurred is consistent with the slow process of resolution of chronic tissue oedema. The outcome in this patient suggests that the prognosis in acne-associated oedema may be better than has been reported,[1,5] and may not always depend on specific treatment with isotretinoin or steroids.

Department of Dermatology,
Stobhill General Hospital,
Glasgow G21 3UW, U.K.

C.Dwyer
D.Dick

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A theoretical model of the blanching response after copper vapour laser treatment of telangiectasia

Sir, It is clear from the literature that superficially detectable vascular malformations,including port wine stains, respond dramatically to treatment using a narrow beam of yellow (578 nm) light from a copper vapour laser, or the 577 nm or 585 nm light from a pulsed dye laser. The typical clinical response when using the copper vapour laser (or indeed any other essentially continuous wave laser)[1] is an immediate blanching of the lesion. The only model proposed which explains the blanching suggests that the result is due to protein denaturing (as in thermally modified egg white).[2] The predictions of this model are inconsistent with the clinical results discussed below. This blanching differs from the purpura effect, which is the clinical response when a pulsed dye laser is used.[3,4] Purpura occurs as a result of agglutination of red blood cells in the vessels.[5]

Histological studies have shown that pulses of light shorter than 0·3 ms cause mainly mechanical damage, due to vaporization of some of the water in the erythrocytes causing abrupt volume expansion of the capillary and consequential rupture of its wall. This mechanical insult is insufficient to permanently damage the vessel,[6] and some degree of thermal damage to the endothelial cells is needed in order to produce a permanent clinical response.[7]

Using the computer-controlled scanner described by Smithies *et al.*[8] with a copper vapour laser, blanching is used clinically as the indicator of thermal damage and thus the appropriate dose. The laser spot has a diameter of 0·4 mm and travels across the lesion leaving a minimally blanched line behind. When 5 W of laser power is used, illumination times used are between 3 and 4 ms. Close clinical observation of the blanching of the skin during the treatment process is possible using the (nearly) full colour vision obtained with rare-earth doped laser eyewear.[9] The skin is seen to blanch at the treatment point, that is within a matter of tens of milliseconds or less. At the same time, or very shortly thereafter, bands of erythema are formed on both sides of the treatment line.

A different response is sometimes obtained when treating ectatic capillaries where a sclerosing agent has been used to collapse larger vessels in the vicinity of the laser treatment. In this situation lines of black or brown are observed.

Theroretical work carried out by Pickering *et al.*[10] showed that when the haemoglobin absorbs the yellow laser light, heat will be conducted from the erythrocytes to the endothelial cells of the vessel walls on a time-scale of approximately 3 ms. The temperature reached by the membrane of the endothelial cells (about 70°C) is sufficiently high to irreparably damage this fundamental cell structure. These models and that of van Gemert *et al.*[11] explain why heating on a time-scale of several milliseconds gives the most selective thermal necrosis.

We now extend this model by proposing that the next step is that the selective permeability of the endothelial cell membrane is irreparably lost. The sudden influx of water carried by the Na+ ions redistributing according to their concentration gradient causes the cells to rapidly enlarge. Because the surrounding dermal structure prevents centrifugal expansion, expansion occurs centripetally, blocking the lumen. When the vessel lumen is almost entirely occluded, the blanching effect is obtained. These steps are shown in Figure 1.

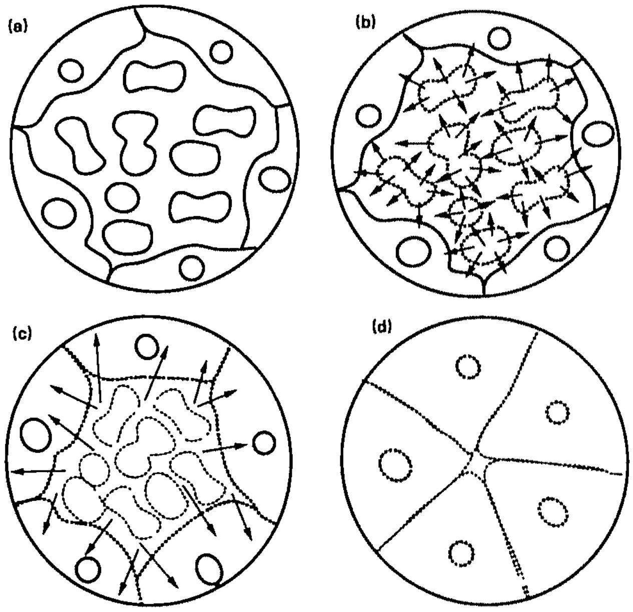

Figure 1. Sequence of thermal damage to ectatic capillary during and immediately after heating by 578 nm laser illumination. (a) Ectatic blood vessel with red blood cells shown. (b) Laser light absorption heats red blood cells, which are damaged. Heat flows outward. (c) Endothelial walls damaged, the osmotic pressure allows the flow of Na+ and water into the cells. (d) Damaged endothelial cells swell to exclude red blood cells, and occlude the lumen.

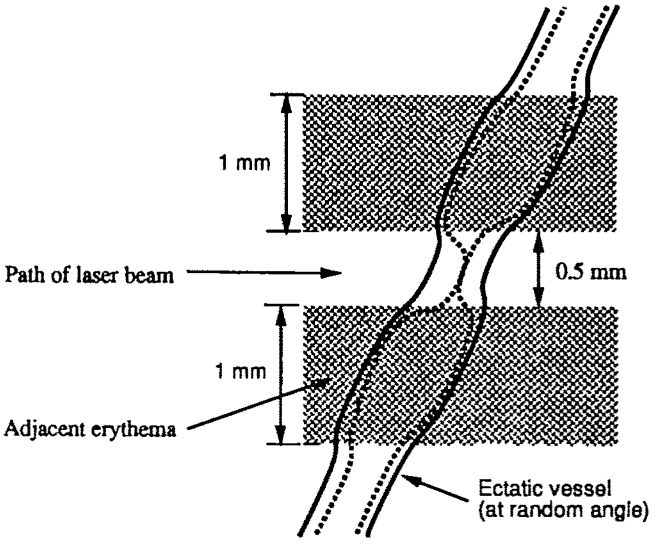

Figure 2. Erythema as a result of laser treatment at 578 nm.

In the case where a sclerosing agent has been used, we propose that some of the escape channels are pre-damaged and swelling, there is haemoconcentration, and the haemoglobin denatures *in situ.* The blood cells are stationary and the colour of the vessel changes to black or brown.

When the laser beam causes thermal damage to the endothelial portion of the vessel structure, vasodilating agents are immediately released by these cells as part of the common series of events occurring during an acute inflammatory process. These agents will dilate the adjacent capillaries, causing a band of erythema on either side of the laser track. The time-scale for this is related to the flow rate of blood in the capillaries (typically 3 mm/s), as shown in Figure 2. These dilated vessels, congested with erythrocytes, present a better target for the returning computer-guided laser beam.

SDC Studio DermoChirurgico-35, L.MARINI
Via Gambini, Trieste, Italy P.H.BUTLER*‡
**Physics Department, University of Canterbury,* D.J.SMITHIES*
Christchurch, New Zealand E.P.WALKER†
†Milford Chambers, 249 Papanui Road,
Christchurch, New Zealand
‡Correspondence: Dr P.H.Butler.

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Acitretin in the treatment of mal de Meleda

SIR, Mal de Meleda is an autosomal recessive keratoderma of the palms and soles. This relatively rare condition is characterized by a diffuse keratoderma which extends on to the dorsal surfaces of the hands and feet, and may affect the knees and elbows. Sometimes the forearms and lower legs are also involved. Other abnormalities which have been described in mal de Meleda include nail thickening or koilonychia and hyperhidrosis. This disorder was originally described on the Dalmation island of Mljet (Meleda).[1] The efficacy of the aromatic retinoids etretinate and acitretin in disorders of keratinization is well established, [2,3] and they usually, but not invariably, produce improvement of palmoplantar keratoderma. In Papillon–Lefèvre syndrome a highly successful response has been documented by several groups.[3-6] In contrast, although hereditary epidermolytic palmoplantar keratoderma (Vörner type) consistently showed a reduction of the hyperkeratosis during retinoid therapy, increased fissuring and blistering accompanied by tenderness always necessitated discontinuation of treatment at an early stage.[7,8] Information on the efficacy of acitretin in other types of palmoplantar keratoderma is limited, and in the rarer disorders such as mal de Meleda information is not yet available. We report the response to acitretin in two patients with mal de Meleda.

A 70-year-old female complained of thickening of the palms and soles which had been present since birth. She had extensive, diffuse hyperkeratosis which extended on to the dorsa of the hands and feet (Fig. 1). The nails showed subungual hyperkeratosis. Seven months prior to presentation the lesions had extended to the distal parts of the lower legs and lower arms in the form of sharply demarcated erythematosquamous plaques. This extension had been noticed following treatment of hypertension with captopril. Although cultures of scrapings had all been negative for fungi, a culture of a skin biopsy grew *Trichophyton rubrum*. A biopsy taken from the margin of the extending lesion on the lower arm revealed epidermal acanthosis and hyperkeratosis, with areas of orthokeratosis and parakeratosis. There was a perivascular mononuclear infiltrate in the dermis. PAS-positive spores were present in the stratum corneum. Acitretin treatment was started at a dose of 35 mg/day and increased to 50 mg/day. Topical therapy with ketoconazole cream twice daily was also commenced. Acitretin treatment resulted in a marked reduction of the hyperkeratosis (Fig. 2); however, blistering and tenderness of the affected skin necessitated reduction of the dosage to 30 mg/day. After 4 months' treatment with acitretin and topical ketoconazole, a marked reduction of hyperkeratosis and a modest improvement of erythema were observed. Unfortunately, severe hair-loss necessitated discontinuation of acitretin, and within 5 days the hyperkeratosis of the palms and soles reappeared.

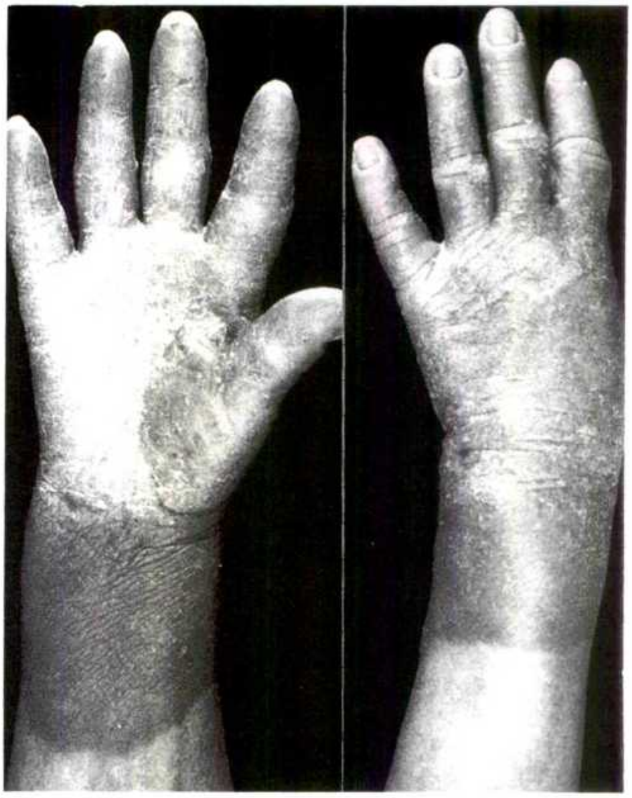
Figure 1. Clinical appearance of the hand in Case 1 before treatment.

A 59-year-old female presented with a hyperkeratotic disorder of the palms and soles, which had been present from birth. On examination there was diffuse hyperkeratosis of the palms and soles, which extended on to the dorsal surfaces of the hands and feet. There was pseudo-ainhum of the 5th finger of the left hand. Mild erythema and moderate palmoplantar hyperhidrosis were additional features. Cultures from the hyperkeratotic areas repeatedly grew *Candida albicans* and *Trichophyton rubrum*. Histopathology of a biopsy taken from the palm showed marked ortho-hyperkeratosis and PAS staining

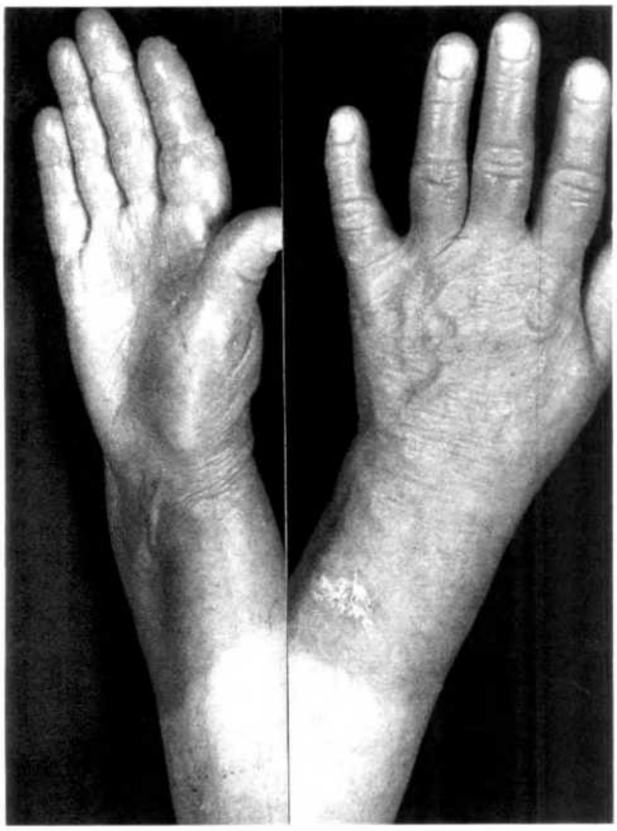

Figure 2. Clinical appearance of the hand in Case 1 after one week of acitretin (35 mg/day).

demonstrated *Candida* hyphae. The hyperkeratotic areas were treated topically with ketoconazole cream, and acitretin was started at a dose of 35 mg/day. After 4 weeks' treatment, the hyperkeratosis was markedly reduced. The erythema, however, was more intense and the patient experienced an unpleasant tenderness of the palms and soles. A dosage of 20 mg/day and 10 mg/day on alternate days was found to be the best compromise. The pseudo-ainhum of the 5th finger had completely resolved after 6 months treatment.

The clinical picture in both patients is compatible with the diagnosis of mal de Meleda. Both patients were severely incapacitated by their skin disorder, and we therefore considered that treatment with acitretin was warranted. Jee *et al.*[9] have previously reported the efficacy of etretinate in mal de Meleda. Treatment with acitretin resulted in a marked reduction of the keratoderma in both cases. Erythema, however, was prominent and became more evident following resolution of the hyperkeratosis. The optimal dosage proved to be between 10 and 30 mg/day. Higher dosages resulted in a marked tenderness of the skin, and in Case 1 blistering was observed at a dosage of 50 mg/day. Improvement occurred within the first month of treatment. In Case 1 discontinuation of acitretin resulted in relapse within days. In various forms of palmoplantar keratoderma cultures of hyperkeratotic material have revealed fungi,[10-12] and fungal infection can be demonstrated in up to 60% of patients with palmoplantar keratoderma.[12] Although it is difficult to estimate to what extent the fungal infection is responsible for the hyperkeratosis and inflammation, the beneficial effect of monotherapy with itraconazole suggests that fungal infection contributes to the symptoms.[11] For this reason, combined treatment with acitretin and topical ketoconazole was prescribed in the present cases.

In conclusion, acitretin can be of benefit in cases of mal de Meleda. However, treatment should be initiated with a relatively low dose (10 mg/day) which should be gradually increased in order to reach an optimal dose which does not result in discomfort or blistering. It is important to be aware of the possibility of concomitant fungal infection, which requires additional antifungal treatment.

Acknowledgment

We wish to thank Charlotte Neger for her secretarial assistance.

Department of Dermatology,
University Hospital Nijmegen,
The Netherlands

P.C.M.van de Kerkhof
R.J.van Dooren-Greebe
P.M.Steijlen

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Widespread folliculitis induced by human granulocyte-colony-stimulating factor therapy

SIR, Colony-stimulating factors (CSFs) are glycoproteins produced by recombinant DNA techniques. They include granulocyte CSF (G-CSF) which primarily stimulates the production and function of neutrophils and to a lesser extent monocytes, and granulocyte-macrophage CSF (GM-CSF) which increases the production of neutrophils, monocytes and eosinophils.[1] CSFs have mainly been used in the amelioration of chemotherapy-induced neutropenia, although they have also been used in the treatment of non-malignant neutropenias,[2] aplastic anaemia, acquired immune deficiency syndrome, myelodysplasia, and to aid recovery following bone marrow transplantation.[3] There have been four previous reports of skin manifestations with this therapy—two cases of leucocytoclastic vasculitis,[4,5] a case of bullous pyoderma gangrenosum,[6] and worsening of pre-existing cutaneous vasculitis in a patient with hairy-cell leukaemia.[7] We report a patient with myelodysplasia who developed a recurrent folliculitis with G-CSF therapy.

A 64-year-old Greek man was referred in April 1991 for treatment of myelodysplasia which had been diagnosed in 1989. At presentation he had a right middle zone lobar pneumonia, oropharyngeal candidiasis, cardiac failure and gastrointestinal bleeding. He was anaemic (haemoglobin 4·3 g/dl), thrombocytopenic (platelets 10×10^9/l) and leucopenic (total white cell count $1{\cdot}4 \times 10^9$/l; neutrophils $1{\cdot}0 \times 10^9$/l). He was treated with repeated blood and platelet transfusions, intravenous cefotaxime and frusemide, and oral erythromycin, fluconazole and amphotericin B suspension.

His condition improved on the above treatment, and subcutaneous G-CSF therapy was started at a dose of 300 μg/day. This was initially well tolerated, with no side-effects, and resulted in a good granulocyte response. Six weeks after starting G-CSF, whilst on 300 μg twice a week, he developed recurrent painful pustular lesions on the face, arms and upper trunk. New lesions appeared within 24 h of each G-CSF injection and lasted 1–2 weeks. On examination there were numerous 2–4-mm-diameter follicular erythematous papules and pustules scattered over the face, scalp, upper chest and arms (Fig. 1). The total white cell count was $19{\cdot}8 \times 10^9$/l, neutrophils $17{\cdot}1 \times 10^9$/l, and platelets 160×10^9/l.

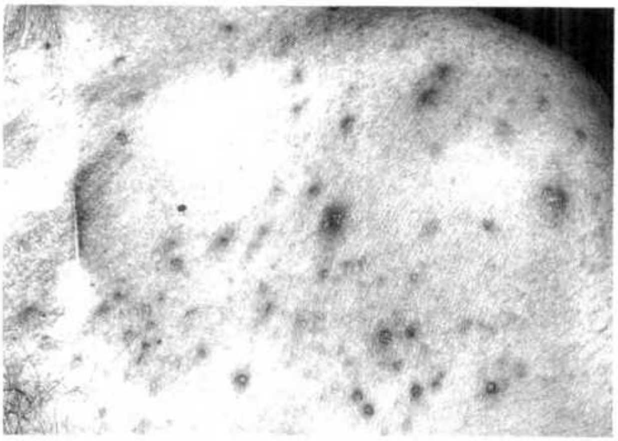

Figure 1. Erythematous papules and pustules over upper trunk.

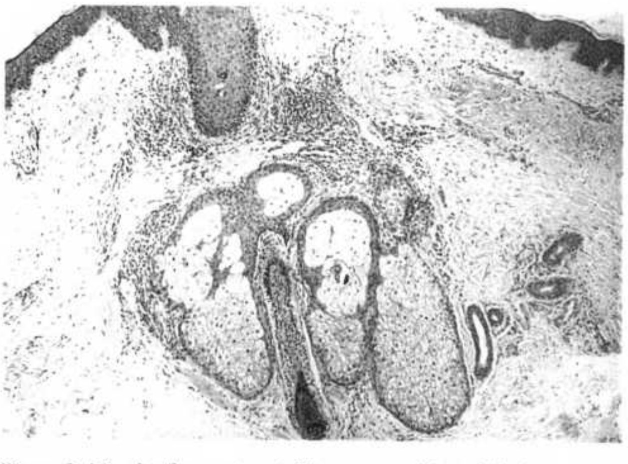

Figure 2. Mixed inflammatory infiltrate around hair follicle.

A skin biopsy showed a mixed inflammatory infiltrate around the hair follicles and eccrine glands (Fig. 2). This consisted principally of lymphocytes and histiocytes with a few neutrophils and eosinophils. PAS, Gram, and Ziehl–Neelsen stains were all negative. Culture of a swab from a pustule produced a scanty growth of coagulase-negative *Staphylococcus aureus*. The G-CSF was slowly reduced to 300 μg once a month which maintains an adequate neutrophil count, and he has had no further episodes of infection. However, he continues to develop new crops of skin lesions within 24 h of each G-CSF injection, which resolve before the next monthly injection.

There have been four previous reports of rashes associated with G-CSF therapy. Wodzinski *et al.*[5] and Welte *et al.*[4] reported leucocytoclastic vasculitis developing in patients with chronic idiopathic neutropenia on G-CSF when the neutrophil count was greater than $1{\cdot}0 \times 10^9$/l. The vasculitis resolved on cessation of the G-CSF. Ross *et al.*[6] reported a 50-year-old woman with small-cell lung cancer who was treated with chemotherapy and G-CSF. She developed vesiculopustular lesions on the hands, the histology of which showed a dermal infiltrate of mature neutrophils, and oedema. A diagnosis of bullous pyoderma gangrenosum was made and the rash resolved with topical corticosteroid treatment after stopping the G-CSF. Glaspy *et al.*[7] described a patient with hairy-cell leukaemia complicated by neutropenia and a histologically confirmed leucocytoclastic vasculitis. After 1 week of G-CSF therapy there was increased size and tenderness of the skin lesions with associated fever. Sweet's syndrome was diagnosed, although a repeat skin biopsy was not performed. The lesions persisted for 2 weeks after the G-CSF was stopped, and then improved.

Drug-induced folliculitis is a well recognized but poorly understood phenomenon. It has been reported with the use of a number of drugs including tetracycline,[8] rifampicin,[9] phenytoin[10] and halogens.[11] A folliculitis has not been previously reported in association with G-CSF therapy. The histology of our case showed a mixed inflammatory infiltrate around the hair follicles consisting principally of lymphocytes and histiocytes, with occasional neutrophils and eosinophils. Our patient is unusually sensitive to G-CSF and requires only monthly subcutaneous injections to maintain an adequate neutrophil count. The development of new skin lesions always occurs within 24 h of G-CSF therapy, thus strongly implicating it in the pathogenesis of the rash.

Department of Dermatology
*and *Haematology,*
The Royal Free Hospital and
School of Medicine,
Pond Street,
Hampstead NW3 2QG, U.K.

L.S.OSTLERE
D.HARRIS
*H.G.PRENTICE
M.H.A.RUSTIN

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Familial multiple pilomatrixomas

SIR, We report a mother and daughter suffering from multiple pilomatrixomas. The occurrence of familial pilomatrixomas is very rare, and as most reported cases involve multiple lesions, a genetic basis must be considered.

Case 1. A 64-year-old woman gave a history of having developed three nodular lesions in early childhood. A lesion on the right temple was excised in 1946, and she was told it was a pilomatrixoma (histology not available). The other lesions, on the right and left upper arms, were asymptomatic and were not treated. On examination in August 1991 the two remaining lesions were hard, angular nodules, located in the subcutaneous tissue, and covered by normal skin. They measured 1 cm in diameter. Physical examination was otherwise normal. The nodule on the right arm was excised, and histology showed typical features of a pilomatrixoma, with prominent calcification.

Case 2. The 31-year-old daughter of Case 1 has, to date, developed a total of eight lesions, of which five have been removed. The first lesion developed on the nape of the neck at the age of 3. Successive lesions then appeared on the left arm, lateral half of the left eyebrow, left leg, left thigh, left popliteal fossa and two on the right upper arm, over a period of 27 years. Clinically a spectrum of presentation was evident. Most of the lesions were subcutaneous with normal overlying skin, and all were hard on palpation. However, the popliteal fossa lesion was eruptive (an exophytic nodule which arose within 3 weeks), and the lower leg and one right upper arm lesion had both discharged yellowish viscous material in the past. Both these lesions were very painful when discharging. Five lesions were removed because they were painful and tender to palpation. Histologically they were typical pilomatrixomas, with calcification and even frank ossification in the thigh lesion.

Neither patient showed any stigmata of myotonic dystrophy, and there was no family history of colonic polyposis. There were no osteomas on skull X-ray, and flexible sigmoidoscopy to the transverse colon was normal.

Pilomatrixoma-like changes have been found in the epidermal cysts of Gardner's syndrome[1] and pilomatrixomas can occur in patients with myotonic dystrophy. In the latter disorder they are often multiple and familial.[2] Eruptive and perforating forms of pilomatrixoma have been described. Eruptive lesions develop rapidly and are usually exophytic, like the popliteal fossa lesion of Case 2. Only seven cases of perforating pilomatrixoma have been reported in the English literature[3] and they represent a form of transepidermal elimination. The history in Case 2 suggested that two of her lesions had perforated.

Moehlenbeck found one pilomatrixoma for every 824 surgical specimens.[4] In a statistical study of 1569 cases of pilomatrixoma he estimated the incidence of multiple tumours as 3·5%. Familial pilomatrixoma in otherwise healthy patients is extremely rare, with only five previous reports in the world literature.[5–8] Rarer still is the occurrence of familial multiple pilomatrixomas, and multiple lesions were present in only two of the reported families. In these two families each affected member had multiple pilomatrixomas. In two other families one member had multiple pilomatrixomas. Moehlenbeck[4] felt that familial occurrence should be considered as an accidental event, in view of the extreme rarity of cases. However, including our cases, there are now six reported families, in three of which multiple lesions have occurred in successive generations, and in two others multiple lesions occurred in members of one generation. A genetic basis should therefore be considered. This seems more plausible if one considers the comparatively high incidence of multiple familial pilomatrixomas in myotonic dystrophy as representing the full expression of the myotonic dystrophy gene, and not merely an associated feature of this autosomal dominant disorder.

Department of Dermatology,
Dryburn Hospital,
Durham DH1 5TW, U.K.

R.J.Hills
F.A.Ive

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Branchial cyst of the neck

Sir, The differential diagnosis of ciliated cystic lesions of the lower neck based upon location and histology may be difficult, because of their similar characteristics.[1] Branchial cleft remnants are a rare condition of which we report a case.

A 20-year-old man presented with a small, asymptomatic, skin-coloured papule situated over the anterior border of the sternomastoid muscle. The lesion had been present since childhood. Two hair shafts protruded from the lesion (Fig. 1). He stated that occasionally small amounts of clear mucoid fluid could be expressed from the lesion by lateral compression. General examination revealed no other abnormalities. Routine laboratory investigations were normal. There was no family history of a similar problem. The lesion was excised under local anaesthesia.

Histology revealed a cystic lesion lined by pseudostratified ciliated columnar epithelium. Beneath the epithelium there was an inflammatory infiltrate with abundant lymphoid tissue.

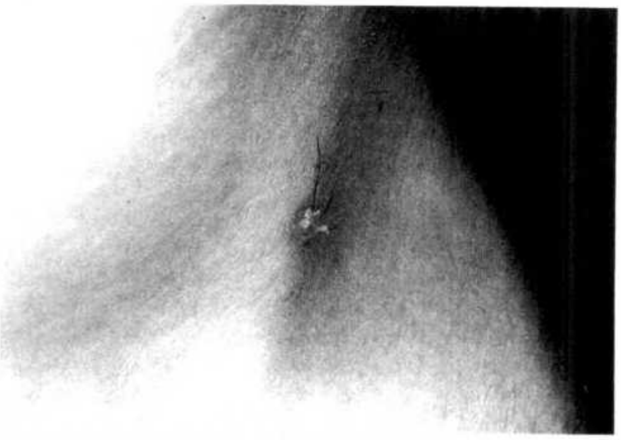

Figure 1. Lesion on the anterior border of the sternomastoid muscle.

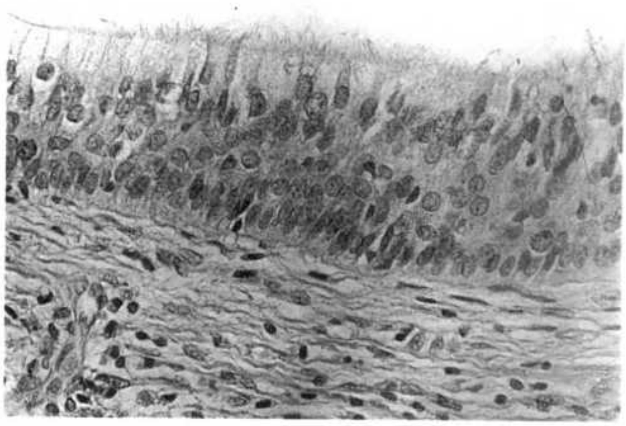

Figure 2. Photomicrograph of cyst lining showing ciliated columnar epithelium with goblet cells (haematoxylin and eosin × 25).

The pseudostratified ciliated columnar epithelium was interspersed with goblet cells which had clear cytoplasm (Fig. 2) and stained positively with diastase resistant PAS. In addition, seromucous glands were present whose acid mucopolysaccharide contents stained with alcian blue.

The differential diagnosis of cutaneous ciliated cysts of the head and neck includes thyroglossal duct cysts, thymic cysts, bronchogenic cysts, and branchial cleft remnants.[1,2] Thyroglossal duct cysts are usually located on the midline near the hyoid bone. They lack goblet cells, and thyroid follicles are often observed in the surrounding periductal connective tissues.[3]

Thymic cysts and sinuses are sometimes lined by ciliated epithelium, but they lack goblet cells, and there is thymic tissue in the walls of the cysts. Their usual location is deep to the anterior border of the sternomastoid muscle anywhere between the angle of the mandible and the manubrium sterni.

The differentiation between branchial cysts and bronchogenic cysts is difficult, because they may have similar characteristics. Some authors consider that the most striking difference is the presence of prominent lymphoid tissue in branchial cysts, as in our case.[4]

Recently Coleman *et al.*[4] assessed ciliated cystic lesions of the head and neck and concluded that many lesions with a similar appearance, especially 'bronchogenic cysts', may in fact have branchial cleft origin.

Clinica Dermatologica,
Ospedale San Paolo,
Via di Rudini 8,
20142 Milano, Italy.

R. Betti
A. Lodi
M. Palvarini
C. Crosti

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British Journal of Dermatology (1992) 127, 196–197.

Book Reviews

Biopsy Pathology of the Skin. N.KIRKHAM (1991). London: Chapman & Hall. Pp. 396. ISBN 0-412-35080-7. Price £59.50.

This latest volume in the series *Biopsy Pathology*, published by Chapman and Hall has been largely written by one author, Nigel Kirkham, with excellent additional contributions on aspects of tropical diseases by Sebastian Lucas. Dr Lucas has also contributed to the chapter on granulomas. The book is aimed at those 'engaged in the everyday task of examining skin biopsies' and Dr Kirkham has 'tried to give a reasonably current account of dermatopathology in everyday practice'.

The first chapter discusses the appearances of the normal skin. This is followed by an interesting and well-written chapter on biopsy methods and strategy for reporting biopsies written by Dr Kirkham and Dr Margaret Price. The remaining chapters are arranged in a more-or-less conventional way and most common inflammatory and neoplastic skin disorders are covered. In addition there is a small section on the skin and AIDS and the final chapter discusses a selection of artefacts seen by the dermatopathologist.

As this book is largely the work of one individual, there is a pleasant uniformity of style and undue repetition is avoided. The illustrative photomicrographs are variable in quality and range from the excellent to the mediocre. There are numerous illustrations including some in colour. Unfortunately many of the illustrations are quite a long way removed from the appropriate reference in the text. This is made much more confusing by the fact that there are no direct references to the illustrations in the text. I feel that this is a mistake. There is no way of knowing, for example, that when one reads about necrolytic migratory erythema being associated with glucagonoma on page 207 that five pages later there is an illustration purporting to show histological features of necrolytic migratory erythema. In other monographs in the series of *Biopsy Pathology*, such as the excellent volume on *Biopsy Pathology of the Lymphoreticular System* by Wright and Isaacson, there are references in the text to specific photomicrographs which illustrate the written descriptions. I would recommend that this practice is followed if a further edition of *Biopsy Pathology of the Skin* is contemplated.

Some of the captions relating to the photomicrographs are perhaps somewhat controversial. On page 163 we are told that the two photomicrographs represent trichoepithelioma and not a nodular basal cell carcinoma. I think some pathologists would find it difficult not to call the lesion illustrated a basal cell epithelioma. Moreover the reason given for the author's suggested diagnosis is that there is focal calcification, a change which not uncommonly occurs in basal cell carcinoma. In other areas of the book, it seems there may have been some inaccuracies in proof-reading. It is difficult otherwise to understand the comment in the legend on page 95 that the cells have small nuclei and indistinct nuclei when in fact the nuclei are very clear, or to know what is meant by the term on page 158 'trichoepithelioma adenoides sebaceum'.

It is easy, however, in any work of this type to find minor inconsistencies and inaccuracies. A lot of work has gone into this volume and there is a lot of useful practical information presented in a well-written style. I wonder, however, whether the fact that *Biopsy Pathology of the Skin* in its size and content falls between the smaller introductions to dermatopathology such as those by MacKie, Burgdorf and others and the larger more comprehensive volumes such as Lever's *Histopathology of the Skin*, and the monograph by McKee, may somewhat limit its appeal.

NEIL SMITH

Clues to Diagnosis in Dermatopathology. A.B.ACKERMAN, M.JACOBSON, P.A.VITALE (1991). Chicago: American Society of Clinical Pathologists Press. Distributed outside USA and Canada by Raven Press, New York. Pp. 428. Illustrations: 736 colour photographs. ISBN 0-89189-296-6. Price $204.

Dr Bernard Ackerman has once again produced a book on dermatopathology which is a delight to look at and to read. The quality of production of this volume and especially the photomicrographs, is superb. The style of the book is somewhat similar to that in previous volumes from this author and colleagues published under the title of '*Differential Diagnosis in Dermatopathology*'.

In this particular monograph, clues to specific histopathological diagnoses are offered although it is acknowledged that many of the 'clues' discussed represent pointers to not one but several possibly unrelated cutaneous disorders. The authors admit that the clues presented are guides and not infallible signs and they hope that new information is presented in this book. A hundred histopathological clues to diagnosis are presented. A single photomicrograph is illustrated on the first page of each section and underneath this is the legend 'What is the clue and what is your diagnosis?'. There follows a discussion of the histological features illustrated, together with further photomicrographs. Occasional references are given at the end of each section but these are by no means comprehensive.

The format of the book makes it a delight to browse through and most individuals will enjoy the self-assessment tests (particularly if these are carried out in private!). Although at the end of the book there is a glossary and index, it would be difficult to use this volume as a reference text.

Many experienced dermatopathologists would accept the importance of many of the clues to which Dr Ackerman and colleagues refer, but with so personal a monograph it is natural that some readers may have slightly different views with regard to the significance of some of the features described. This of course is to be expected and the spirit of a constructive controversy has long been championed by Dr Ackerman himself.

It is a pity that in the section on desmoplastic trichoepithelioma no mention is made of the important distinction of this lesion from microcystic adnexal carcinoma, although syringoma and morphoeic basal cell epithelioma are mentioned. One gets a feeling that with some of the clues one needs a little more information than that given to the reader at the outset. For example, both Clue 75 and Clue 96 are photomicrographs of a clear cell lesion with prominent vasculature. The answers to the clues however are quite different, one being 'profuse vascularity in a clear cell neoplasm is a clue to renal cell carcinoma metastatic to the skin' and the other is 'profuse vasculature in a neoplasm replete with mucin is a clue to myxoid liposarcoma'.

The discussion relating to Clue 10 is somewhat controversial. The authors state that guttate parapsoriasis and digitate dermatosis are simply different names for what appears to be a single pathological process. Some people use the term 'guttate parapsoriasis' to be synonymous with pityriasis lichenoides while recognizing that digitate dermatosis is a variety of chronic superficial dermatitis and quite a benign condition. The authors go on to state that they have come to recognize this condition (i.e. guttate parapsoriasis/digitate dermatosis) as one of the expressions of mycosis fungoides. If at the outset of this section the reader is somewhat confused about the terminology of parapsoriasis I feel that he is not going to be very much wiser having read this section.

As mentioned above, however, in a book of this style it is easy to find things to criticise. Dr Ackerman and his co-authors, Drs Jacobson and Vitale, should be congratulated on producing an attractive, informative, stimulating and fun book. For some students of histopathology, the price of the book may be something of a deterrent given that some of the more standard and comprehensive works on cutaneous histopathology are available at a comparable price. Others, however, may find this book an excellent investment and will be already saving to buy the sequel that is planned!

NEIL SMITH

British Journal of Dermatology (1992) 127, 198.

News and Notices

Announcing BEES—the British Epidermo-Epidemiology Society

The inaugural meeting of the BEES group was held on the 24 April 1992 at St John's Dermatology Centre, St Thomas' Hospital. Four stimulating talks were given on a wide range of topics including butcher's warts (Dr Martin Keefe), early predictors of adult disease (Dr Keith Godfrey), repeatability of physical signs (Dr Hywel Williams) and melanoma in families (Dr Julia Newton). Sessions were chaired by Dr John Savin, Professor Jean Golding, Professor Rod Hay and Dr Nick Simpson, and the audience was comprised of dermatologists, epidemiologists, statisticians and public health physicians, a mix which led to very useful interdisciplinary discussions from the floor.

The society aims to promote a high standard of epidemiological research into all forms of skin disease, with emphasis on an interdisciplinary approach. The next meeting is scheduled for the 22 January 1993 at St John's Dermatology Centre and will be announced formally through the dermatology and epidemiology societies.

Anyone wishing for more information about the society and future meetings should contact: Dr Hywel Williams, Research Fellow in Dermato-Epidemiology, St John's Dermatology Centre, St Thomas' Hospital, Lambeth Palace Road, London SE1 7EH, U.K.

The Skin Disease Research Fund

The fund invites further applications for grants. The deadline for submissions is 4 September 1992 and an indication of the result will be given during November. Only the Fund's own forms will be accepted and these may be obtained from: The BAD Office, 3 St Andrew's Place, London NW1 4LB. Telephone 071-935-8576.

8th Annual Symposium of the British Society of Paediatric Dermatology, 6–7 November 1992, Brighton, U.K.

The 8th Annual Symposium of the British Society of Paediatric Dermatology will take place in Brighton on 6–7 November 1992. The topic of the Symposium this year is Infectious Diseases in Children. For further details, please contact: Dr Charles Darley, Brighton General Hospital, Elm Grove, Brighton, East Sussex, U.K.

17th Annual Hawaii Dermatology Seminars, February 1993, Hawaii

The 17th Annual Hawaii Dermatology Seminars will take place on Hawaii in February 1993. The Seminars and venues are as follows: Pre-Seminar (Financial and Business Matters), 16–19 February 1993, Manele Bay Hotel/The Lodge at Koele, Lanai, Hawaii; Hawaii Dermatology Seminar, 19–24 February 1993, Hyatt Regency Waikoloa, Big Island, Hawaii; Post-Seminar Meeting, 19–24 February 1993, Westin Surf Resort, Kauai, Hawaii. For further information and registration contact: Dr Henry Roenigk, Department of Dermatology, Northwestern University, 303 E. Chicago Avenue, Chicago, IL 60611, U.S.A. Telephone 312-908 8173. Fax 312-908-0664.

British Journal of Dermatology (1992) 127, 199–204.

REVIEW

Churg–Strauss syndrome

R.A.SCHWARTZ AND J.CHURG*

*Departments of Dermatology and *Pathology, New Jersey Medical School, Newark, New Jersey, U.S.A.*

Accepted for publication 8 May 1992

Churg–Strauss syndrome (CSS) is a clinicopathological entity in which prominent eosinophilia with asthma or allergic rhinitis is associated with a systemic vasculitis.[1–6] It is a disorder in which the cutaneous findings may lead to an early, potentially life-saving, diagnosis. The organs most evidently involved clinically are the heart, lungs, kidney, skin, and peripheral nerves. Some of the frequently seen cutaneous lesions may show the characteristic granuloma, facilitating diagnosis.

History

The original description of this disorder as a distinct clinicopathological entity was by one of us (JC) and Lotte Strauss in 1949,[1,2] in a study of case material mostly derived from autopsies at the Mount Sinai Hospital in Manhattan. Thirteen patients were reported who had a uniform clinicopathological syndrome of severe asthma, fever, and hypereosinophilia, together with severe vascular involvement in various organ systems, frequently including skin. A number of reports of allergic states with vascular lesions resembling those of periarteritis nodosa had been documented previously,[2,7–9] with a clinical description of this allergic granulomatosis by Rackemann and Greene in 1939.[8] The cutaneous clinical and histological findings were described in detail by Strauss, Churg, and Zak in 1951.[3]

Incidence

This is an uncommon syndrome, the diagnosis of which may be difficult on histological grounds, as the three histological components may not occur in the same organ at the same time[4] and may be found together in life in only a minority of patients.[5, 6,10–12] In a random collection of 745 patients with the eight major types of vasculitis, only 10 (1·3%) had Churg–Strauss syndrome.[10] However, the syndrome may be underdiagnosed.[5]

Aetiology

The aetiology of this disorder is unknown. Whether it is best categorized as an allergic disorder or a vascular disease is unclear. We believe that the critical element in the pathogenesis of CSS is a proliferation of activated eosinophils, a variation in normal tissue reaction, producing a cascade of aberrant tissue responses. IgE-containing circulating immune complexes have been demonstrated in patients with CSS and may be responsible for the vasculitis.[13] However, there is evidence that anticytoplasmic antibodies (p–ANCA) are usually present in these patients, suggesting the possibility of autoimmunity.[14] While periarteritis nodosa is often associated with hepatitis B infection, this is not the case in Churg–Strauss syndrome; only one patient has been reported with antibodies to both hepatitis B virus and human immunodeficiency virus.[15]

Epidemiology

Churg–Strauss syndrome tends to have its onset between 20 and 40 years of age, affecting men and women almost equally, but with a slight male predominance.[6]

Clinical features

Cutaneous CSS

A cutaneous eruption is seen in 70% of patients.[5,16] It may appear as an admixture of macules, papules, nodules and sometimes papulovesicles occurring in crops. Lesions tend to appear most commonly on the

Correspondence: Professor R.A.Schwartz, Department of Dermatology, New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103-2714, U.S.A.

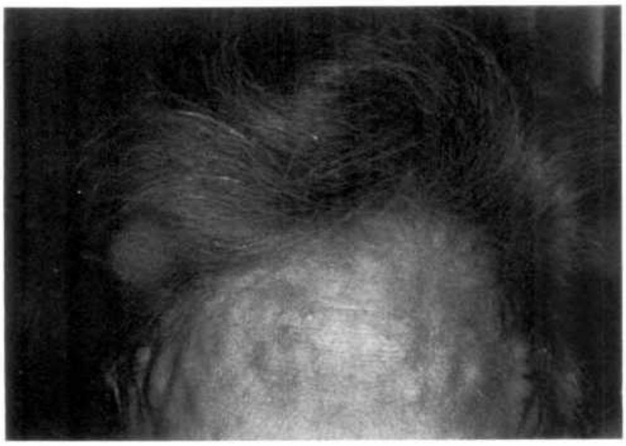

Figure 1. Cutaneous nodules on the scalp (courtesy of Dr Joseph N. Grizzanti).

extensor and flexor surfaces of the extremities, with the skin of the back and abdomen also frequently involved. Nodules usually occur on the extremities and scalp (Fig. 1) and less commonly on the trunk. The lesions may be divided into three categories: erythematous maculopapules resembling erythema multiforme, haemorrhagic lesions ranging from petechiae to extensive ecchymoses, and cutaneous and subcutaneous nodules.[3] The purpuric lesions are often palpable. Some of the haemorrhagic lesions may show local necrosis or be associated with urticarial weals similar to those of Henoch–Schoenlein purpura. Ulcers (Fig. 2) are usually due to arteritis of dermal vessels, but are sometimes the result of breakdown of nodules. The subcutaneous lesions are the most persistent, resolving with scarring within 2 or 3 months. They are the most distinctive, often being tender, and histologically showing the characteristic granuloma of Churg–Strauss syndrome.[5] The histology of the other lesions is most commonly a leucocytoclastic vasculitis. In addition, two non-specific findings, livedo reticularis of the lower legs, and facial or periorbital oedema, may be evident.[16]

Early extracutaneous CSS

CSS usually begins with an allergic rhinitis, often associated with eosinophilia.[5,6] There is often no history or family history of atopy. The rhinitis may be severe, with pansinusitis demonstrated radiographically. Later, asthma develops, beginning in a mild form and later becoming more severe, with transient pulmonary infiltrates (Loeffler's syndrome) or more permanent infiltration producing a clinical picture of chronic eosinophilic pneumonia. In some patients the severe asthma may disappear spontaneously when signs of vasculitis appear.

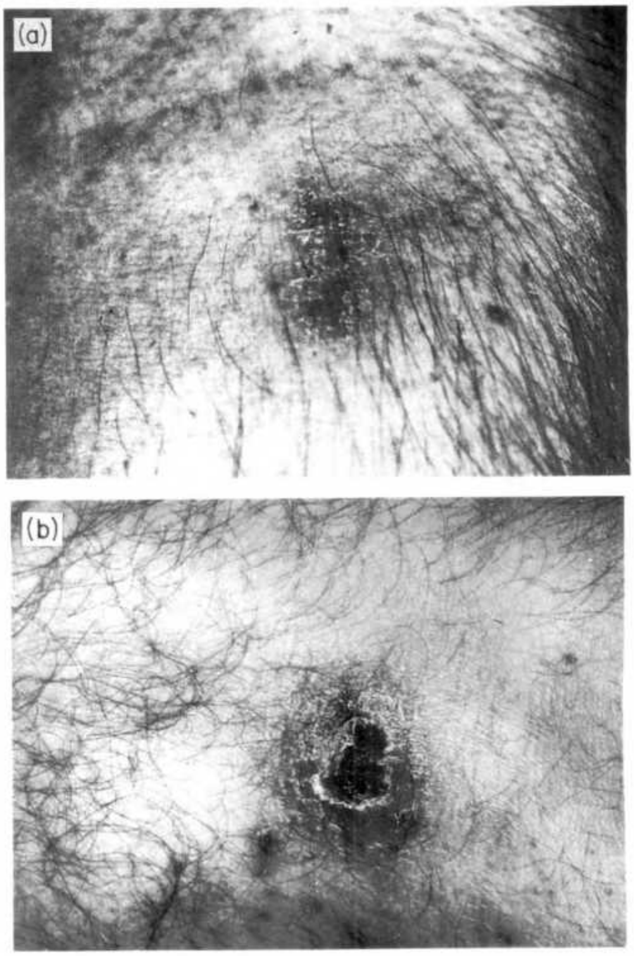

Figure 2. (a and b) Cutaneous ulceration (courtesy of Dr Mark Lebwohl).

Eosinophilic infiltrative CSS

There may be an eosinophilic infiltration of the lung or of the gut. Mucosal involvement of the gut may produce bleeding and diarrhoea, whereas submucosal infiltration may result in nodular masses producing obstructive symptoms. Serosal infiltration may result in peritonitis and ascites, with ascitic fluid containing numerous eosinophils.

Vasculitic CSS

When this phase occurs, the patient may have weakness and malaise with fevers. Hyperpyrexia may be impressive, with marked fluctuations. Cramps in the calves may also occur. The exact symptomatology depends upon the organ system affected by the vasculitis, with recovery often complete, except when the vasculitis involves the peripheral nerves.

Major extracutaneous targets of CSS are the heart, lungs, nervous system, gut, and kidneys. The first signs may be on the electrocardiogram, the presence of a pericardial friction rub, or an atrial gallop. In the pre-steroid era, cardiac involvement often proved fatal, usually due to eosinophilic infiltration of the myocardium or from vasculitis affecting the coronary arteries.[17] Mitral regurgitation is common in CSS.[17,18] Pulmonary involvement may be manifest as transient infiltrates or by a more diffuse and even massive pattern of chronic eosinophilic pneumonia. Alveolar haemorrhage may produce respiratory failure. The neurological involvement is most typically a mononeuritis multiplex. However, cerebral haemorrhage or infarction is a common cause of death in CSS, due to either hypertension or to vasculitis. Gut involvement may be manifest by perforation, peritonitis, obstruction, or cholecystitis. An acute acalculous cholecystitis may be the first sign of this systemic vasculitis.[19] Renal involvement is characterized by focal segmental glomerulonephritis, a major problem in some untreated patients. One recent series noted renal involvement in 84% of patients, in some of whom it was severe.[20]

CSS may also involve the urogenital tract, the joints, muscles, eyes, and reticuloendothelial system. An obstructive uropathy may occur, as a result of urinary tract inflammation. A migratory arthritis may be noted, as may myositis, and a wide variety of ocular changes. Splenomegaly and transient lymphadenopathy may also occur.

Pathology

The three major types of lesions in CSS are eosinophilic infiltration, granuloma formation and necrotizing vasculitis.[6,11] In CSS the eosinophilic infiltrate progresses into granuloma formation, beginning with necrosis within a densely packed cluster of eosinophils. The necrotic core enlarges with inclusion of collagenous stroma, and epithelioid and giant cells appear around the core to produce a palisading formation several cells thick (Figs 3, 4 and 5). This eosinophilic granuloma may heal spontaneously, or after therapy. The vasculitis mainly affects small arteries, with fibrinoid necrosis (Fig. 6), many eosinophils, and a tendency to granuloma formation in and near the vascular wall (Fig. 7). Larger vessels, including veins may also be involved, with inflammatory changes also producing thrombosis and sometimes aneurysm formation. At times a typical leucocytoclastic vasculitis may be evident in the small vessels, with a predominance of neutrophils. Sometimes a skin biopsy of a non-blanching macular erythema may show only a non-specific perivascular infiltrate of numerous eosinophils and mononuclear cells, without evidence of vasculitis.[21]

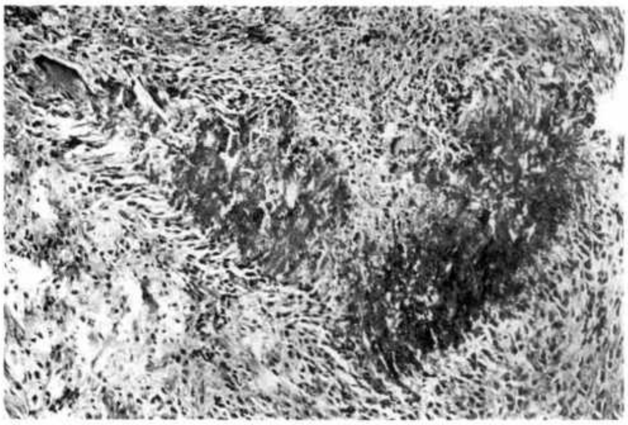

Figure 3. Early cutaneous granuloma showing necrosis of eosinophils in the centre and inflammatory cells (histiocytes and multinucleated giant cells) around the periphery. (Same case as in Fig. 1.)

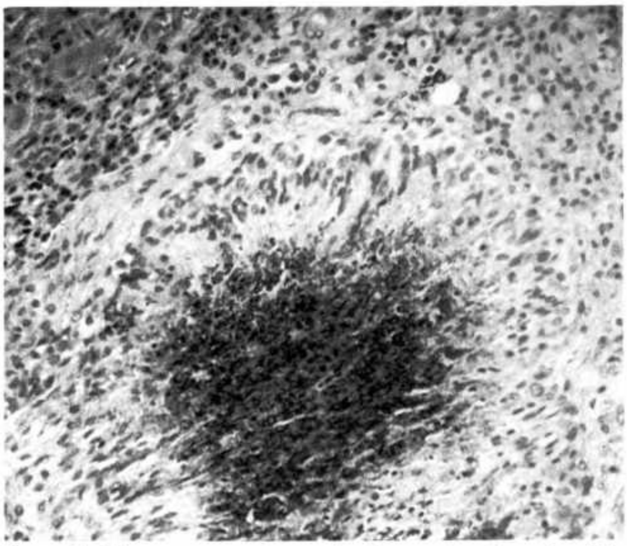

Figure 4. Cutaneous granuloma, showing a strongly eosinophilic centre consisting of disintegrated eosinophils, surrounded by palisading histiocytes.

Laboratory findings

Eosinophilia is a hallmark of CSS.[5] However, there may be eosinophilic infiltration of tissue without blood

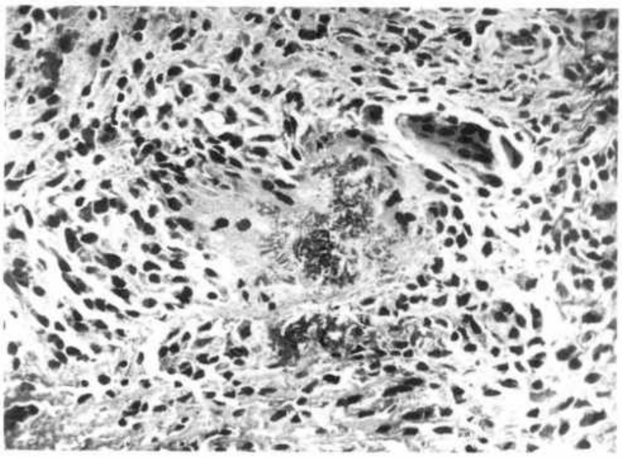

Figure 5. Cutaneous granuloma, regressing. There are remnants of necrosis, and early fibrosis in the centre, surrounded by many layers of histiocytes, and a few multinucleated giant cells.

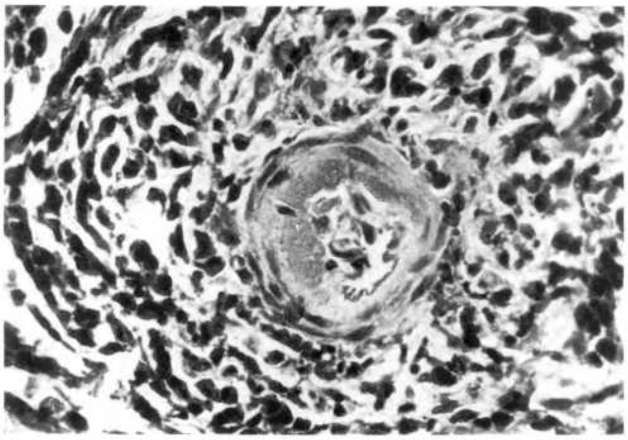

Figure 6. Early (pre-granulomatous) vasculitis. Small artery showing endothelial detachment, a layer of fibrinoid necrosis (left side and top of vessel wall), and intense eosinophilic perivascular inflammation.

eosinophilia. Steroid therapy or wide fluctuations in eosinophil counts may produce a seemingly normal result in some CSS patients. Eosinophilia may appear at any time in the course of CSS. In the original report of Churg and Strauss,[2] all the patients had peak eosinophil counts exceeding 5×10^9/l. IgE levels may be elevated. IgE-containing circulating immune complexes have been demonstrated,[13] as well as anticytoplasmic antibodies (p-ANCA).[14]

Cardiac involvement in CSS may be demonstrated by echocardiography,[18] which may show mitral regurgitation caused by diffuse myocardial fibrosis. A scintigram with ^{111}In-antimyosin may facilitate diagnosis and assessment of the myocardial damage.[22]

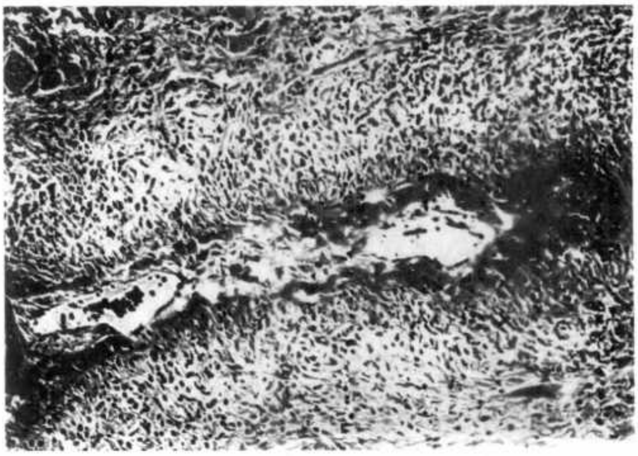

Figure 7. Granulomatous vasculitis. A longitudinal section of an artery demonstrating fibrinoid necrosis of the wall and a multilayered perivascular zone of palisading histiocytes. Multinucleated giant cells are seen in the upper left corner.

Diagnosis

This is a systemic disease. Encouraging a shift of emphasis to clinical diagnosis, Lanham *et al.*[5] noted three criteria for diagnosis: asthma, peak peripheral blood eosinophil count in excess of $1{\cdot}5 \times 10^9$/l, and the characteristic systemic vasculitis involving two or more extrapulmonary organs. The latter requires histological analysis, for which the skin and kidney are the most useful sites. This may not be a rare disease, if given proper diagnostic consideration.[23]

Differential diagnosis

The type of granuloma seen in Churg–Strauss syndrome, the Churg–Strauss granuloma, may be seen in other disorders, including rheumatoid arthritis, Wegener's granulomatosis, periarteritis nodosa, systemic lupus erythematosus, subacute bacterial endocarditis, lymphoproliferative disorders and chronic active hepatitis.[24] However, the specific feature of allergic granuloma is a core of necrotic eosinophils. The presence of eosinophils in a leucocytoclastic vasculitis should alert the dermatopathologist to the possibility of CSS. Similarly, the presence of perivascular eosinophils may suggest consideration of CSS in an appropriate clinical setting.[21]

The cutaneous lesions must be distinguished from drug eruptions, leucocytoclastic vasculitis, cutaneous lymphoma, syphilis, periarteritis nodosa and Wegener's granulomatosis. The latter two diseases may show similar cutaneous lesions to CSS.[16,25,26] However, in periarteritis nodosa cutaneous and subcutaneous nodules are much less likely.[16]

Course

CSS has a variable course, but it is generally favourable with appropriate treatment. A small percentage of patients have prolonged and recurrent disease despite therapy.

Complications

The most frightening prospect in CSS is sudden death, which may occur for a variety of reasons.[27] Atrophic scars are the main complication of old subcutaneous nodules,[6] but they do not always occur. The major complications of the vasculitic phase are hypertension and permanent peripheral nerve damage.[5] Myocardial fibrosis may be so severe as to require valve replacement.[18] Two patients were recently reported with cholinesterase deficiency and CSS.[28] Whether related to CSS, its treatment, or occurring as an incidental association, cholinesterase deficiency should be considered prior to use of therapy which might be affected by such a deficiency.

Treatment

Steroids are the treatment of choice; at times early therapy can be lifesaving.[5,6] The usual initial dose is prednisone 60–100 mg daily. We recommend simultaneous treatment with an immunosuppressant, the most popular of which is cyclophosphamide, to reduce or prevent irreversible major organ damage.[6] In a severe acute situation, Lanham and Churg[6] recommend concurrent boluses of intravenous cyclophosphamide 10 mg/kg and 1–2 mg/kg of methylprednisolone. The former may be repeated after 1 or 2 weeks if needed. Once remission has occurred, they replace cyclophosphamide with azathioprine 2·5 mg/kg/day and continue this for 6–12 months of complete remission. Chlorambucil is also effective, and plasma exchange may be added to the regimen of steroids and immunosuppressants.[29] Pulse intravenous cyclophosphamide may be useful.[30] Occasionally, heart transplantation may be necessary for heart failure in CSS.[31]

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British Journal of Dermatology (1992) 127, 205–211.

Preferential adherence of T lymphocytes and neutrophils to psoriatic epidermis

J.N.W.N.BARKER, R.W.GROVES, M.H.ALLEN AND D.M.MACDONALD
Laboratory of Applied Dermatopathology, United Medical and Dental Schools, Guy's Hospital, London, U.K.

Accepted for publication 28 February 1992

Summary T lymphocytes and neutrophils accumulate in psoriatic epidermis. To determine whether the epidermis plays an active role in this process through the production of cellular adhesion factors, leucocyte adherence to lesional psoriasis was compared with normal skin in a modified frozen-section adhesion assay. Lymphocyte and neutrophil suspensions were prepared by standard Ficoll–Hypaque techniques from peripheral blood of normal volunteers and overlaid on to glutaraldehyde-fixed 8-μm cryostat sections of skin. Adhesion of phorbol ester-activated T lymphocytes to the epidermis was significantly greater in psoriasis compared with normal skin ($P<0{\cdot}01$). Adhesion was absent (a) at 7°C, (b) in the presence of EDTA and (c) in the absence of lymphocyte activation. Immunostaining demonstrated that all adherent lymphocytes were CD3+ve (i.e. T cells). Likewise, neutrophils adhered more prominently to psoriatic epidermis. Adhesion was most prominent at the tips of dermal papillae, corresponding to areas of maximal intercellular adhesion molecule-1 (ICAM-1) expression. Both neutrophils and lymphocytes adhered to dermal papillary vascular endothelium. These studies provide functional data that psoriatic epidermal cells are actively involved in leucocyte adherence. The distribution of adhesion suggests that both ICAM-1-dependent and independent mechanisms are involved.

Leucocytes normally circulate in the peripheral blood in a non-adherent state but at specific sites and under certain conditions they adhere to, and migrate through the blood vessel wall. This process is common to a variety of events involving leucocytes, including accumulation at sites of inflammation and lymphocyte homing to high endothelial venules (HEV) in lymph nodes. These processes are directed by highly specific receptor/ligand interactions between surface adhesion molecules on leucocytes and their ligands on specialized vascular endothelium. Control mechanisms for such processes, central to most leucocyte functions, include (a) conformational changes in surface expression upon lymphocyte activation, (b) translocation of cytoplasmic proteins to the cell surface induced by inflammatory mediators and (c) transcriptional regulation by cytokines.[1]

Psoriasis is a common inflammatory cutaneous disorder characterized by hyperproliferation of the epidermis and by an inflammatory infiltrate comprising T lymphocytes and neutrophils.[2] These cells accumulate both in the dermis, which in normal skin is composed mainly of collagen, fibroblasts and vascular endothelium, and in the epidermis, which consists primarily of keratinocytes but also includes specialized dendritic antigen-presenting cells (Langerhans cells). Interactions between infiltrating leucocytes and cutaneous structures, particularly keratinocytes, are thought to play a critical role in the pathophysiology of psoriasis via the production of pro-inflammatory cytokines and epithelial growth factors including tumour necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8 and transforming growth factor-α (TGF-α).[3,4]

Functional studies designed to delineate mechanisms of leucocyte recruitment in psoriasis have to date focused on interactions between lymphocytes and dermal vascular endothelium. It has been demonstrated that T lymphocytes but not B cells preferentially adhere to dermal vessels in psoriasis compared with normal skin, a mechanism involving multiple receptor/ligand pathways.[5] These data indicate an active role for dermal endothelium in the recruitment of leucocytes into psoriatic dermis. Although *in vitro*[6] and immunohistochemical[7,8] studies suggest that epidermal accumulation of leucocytes involves lymphocyte function associated antigen-1 (LFA-1)/intercellular adhesion molecule-1

Correspondence: Dr J.N.W.N.Barker, Department of Dermatology, Guy's Hospital, London SE1 9RT, U.K.

(ICAM-1) interactions, such mechanisms have not been examined functionally.

In this present study a modified frozen-section adherence assay[9,10] was developed to determine whether leucocytes adhere to lesional epidermis. We report that in contrast with normal skin, neutrophils and T lymphocytes, but not B cells, preferentially adhere to psoriatic epidermis.

Methods

Collection and preparation of tissue

Adult patients presenting to the dermatology clinic at Guy's Hospital with typical chronic plaque psoriasis vulgaris were recruited to the study ($n=8$; five male, three female, age range 24–43 years), after ethical permission was obtained. None of the patients had received topical or systemic therapy for 4 weeks prior to inclusion in the study. Biopsies were obtained from the edge of psoriatic plaques under local anaesthetic. Site-matched, normal skin ($n=8$) was obtained from age-matched volunteers, who had no history of psoriasis. All biopsies were immediately snap-frozen in liquid nitrogen-cooled isopentane and stored at −70°C until required. On the day of the adhesion assay (see below) 8 μm cryostat sections were cut, placed on to uncoated glass slides and fixed for 5 min in 2% glutaraldehyde.

Leucocyte preparation

Heparinized venous blood (10 ml) was obtained from young healthy volunteers, mixed with 10 ml Dulbecco's modified Eagle's medium (DMEM)/10% heat-inactivated fetal calf serum (FCS) and layered over a 12-ml Ficoll–Hypaque (Lymphoprep, Nycomed, Oslo, Norway) cushion. After centrifugation (30 min at 260 *g*) the lymphocyte-rich layer was removed by pipette, washed three times in DMEM/10% FCS and activated with 50 nM 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) (Sigma Chemical Co., St Louis, MO, U.S.A.) for 30 min in DMEM. Washing steps were then repeated and the lymphocytes resuspended in DMEM medium to a final concentration of 3×10^6 cells/ml. By cytometry, lymphocyte purity was 90%.

For neutrophil isolation 10 ml heparinized whole blood was mixed with 2·5 ml 6% dextran, incubated at room temperature for 45 min and the leucocyte-rich upper layer removed and layered on to an equal volume of Lymphoprep. After centrifugation (40 min at 400 *g*) the neutrophil pellet at the base of the tube was equilibrated with lysis buffer (ammonium chloride 0·8%, potassium HCO_2 1%, EDTA pH 7·2), incubated on ice for 10 min, washed and resuspended in DMEM/10% FCS.

Leucocyte adhesion assay

After fixation, each 8 μm cryostat section on the glass slide was circled using a hydrophobic marker pen and washed with Ca^{2+}/Mg^{2+}-containing medium. Fifty microlitres of lymphocyte suspension (number of cells $=1\cdot5\times10^5$) or neutrophil suspension (number of cells $=1\times10^6$) was then overlayed on to each section, and the slides incubated for 45 min at 37°C/5% CO_2 with constant agitation. Non-adherent cells were removed by dipping glass slides 10 times in DMEM, the sections stained with toluidine blue, dehydrated through graded alcohols, and mounted in DePeX (BDH). Adherent lymphocytes and neutrophils were clearly distinguishable as dark cells above the plane of the section, which was of paler blue. Cells were quantified using a calibrated eyepiece graticule at ×40 magnification (Leitz light microscope) and cells counted in 100 consecutive unit fields. Statistical analysis was performed using the Student's *t*-test.

Because the number of adherent cells varied from experiment to experiment all results illustrated (see below) were performed at identical times using the same

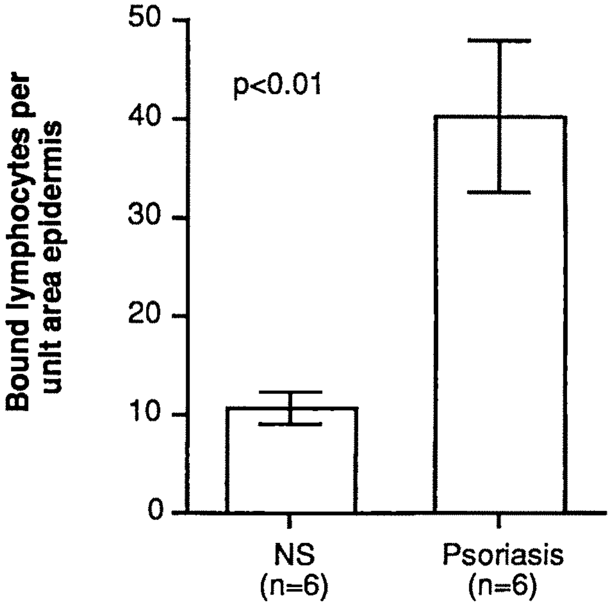

Figure 1. Quantification of TPA-activated peripheral blood lymphocyte adherence to the epidermis of normal skin (NS) and psoriasis. Significantly greater numbers adhere to psoriatic epidermis ($P<0\cdot01$).

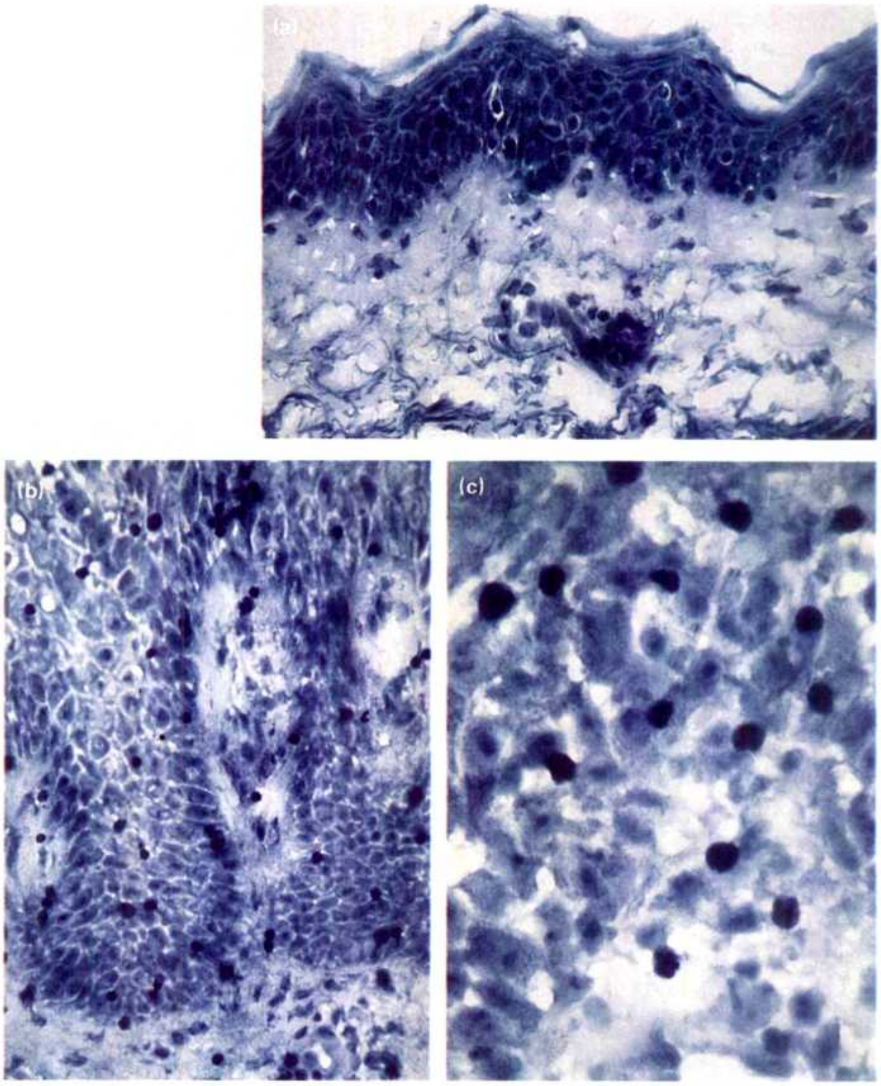

Figure 2. Photomicrographs of TPA-activated lymphocyte adherence to epidermis (a) normal skin (× 100), (b) psoriasis (× 150). Adherent lymphocytes are clearly visible as dark round cells above the plane of the tissue section. (c) Psoriasis at high magnification (× 250) demonstrating prominent adhesion at tip of dermal papilla.

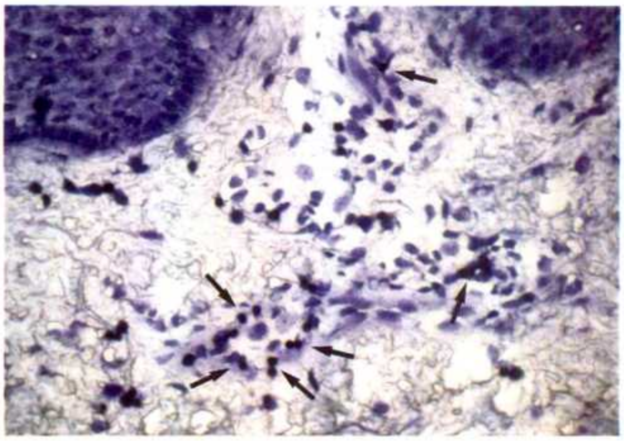

Figure 3. Photomicrograph of upper dermal blood vessel in psoriasis demonstrating adherence of lymphocytes to endothelium (× 100).

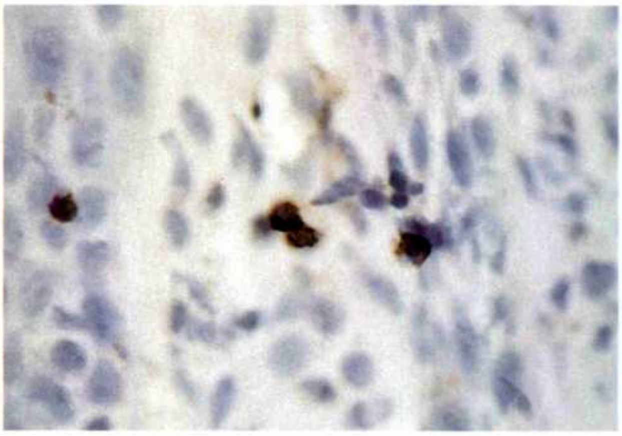

Figure 4. Photomicrograph of dermal papilla in psoriasis demonstrating CD3 expression by lymphocytes adherent to epidermis and vascular endothelium (× 200).

source of lymphocytes, under identical conditions. All experiments were performed a minimum of four times. All washing steps were performed in Ca^{2+}/Mg^{2+}-containing media. In some experiments 0·5 mM EDTA was added to the incubation medium.

Lymphocyte phenotyping

After completion of the incubation stage of the adhesion reaction (see above), each section was washed as before and fixed in cold acetone for 5 min. Sections were then incubated for 30 min with murine monoclonal antibodies to either CD3 (T cells)(Leu 1; Beckton-Dickinson—dilution = 1:20) or to CD22 (B cells)(Dako-B-cell; dilution = 1:20), and labelled cells visualized using a standard indirect immunoperoxidase reaction with 3′-3′-diaminobenzidine as chromogen.[11] Adherent B and T cells could then be observed microscopically.

Phenotype of lymphocytes in overlaid suspension was determined by the same technique on cytospin preparations.

Immunohistochemistry

Serial 5 μm cryostat sections of those used for the adhesion assay (see above) were cut from each biopsy of psoriatic ($n=6$) and normal skin ($n=6$), air-dried on to silane-coated glass slides and fixed in cold acetone for 10 min. Each section was then incubated with murine monoclonal antibody RR1/1 to ICAM-1 (gift of Dr T.A.Springer; dilution = 1:1000). Labelling was visualized by using a peroxidase-antiperoxidase technique.[11] Controls included omission of the primary antibody and substitution with an antibody of the same IgG isotype, but of irrelevant specificity.

Results

Optimal conditions

Preliminary experiments were performed to determine optimal conditions for leucocyte adherence to psoriasis frozen-sections (at least four separate assays for each variable). Maximal adhesion occurred between 30 min and 1 h incubation. At 2 h adhesion was almost completely absent. Adhesion was assessed at 37, 24 and 7°C: maximal adhesion occurred at 37°C and total inhibition at 7°C. Fixation of tissue sections in 2% glutaraldehyde for 5 min before the incubation step consistently increased the number of adherent leucocytes.

Lymphocyte adhesion

Prominent epidermal adhesion was present in psoriasis; mean number of lymphocytes per high-powered field = 40·3 (SEM = ± 7·7). This was significantly different compared with normal skin, mean number of lymphocytes per high-powered field = 10·8 (SEM ± 1·38), $P<0{\cdot}01$ (Figs 1 and 2). Adherent lymphoctes were most numerous in the epidermis overlying the tips of dermal papillae, although lymphocytes were also observed at other sites, including the bases of the rete ridges. In addition, lymphocytes adhered to dermal structures, particularly to papillary vascular endothelial cells (Fig. 3) as previously described. Adhesion was lymphocyte activation-dependent and Ca^{2+}/Mg^{2+}-dependent, as binding of non-TPA-treated lymphocytes did not exceed basal levels, and 0·5 mM EDTA abolished adhesion.

Immunostaining of acetone-fixed sections demonstrated that 90% of adherent lymphocytes expressed CD3 (Fig. 4) and none expressed CD22. In contrast the T-cell to B-cell ratio in cytospin preparations of lymphocyte suspension was 5:1. Consistently fewer adherent cells were observed after the immunostaining procedure compared with results obtained from the standard protocol.

Neutrophil adhesion

Significantly greater numbers of neutrophils were found

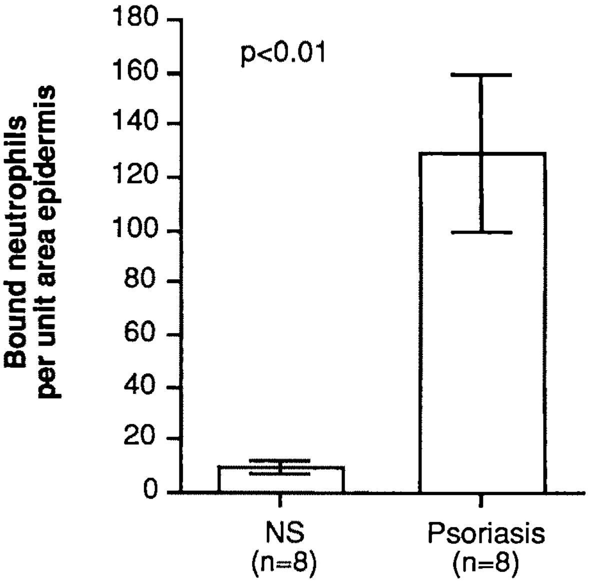

Figure 5. Quantification of peripheral blood neutrophil adherence to epidermis of normal skin (NS) and psoriasis. Significantly greater numbers adhere to psoriatic epidermis ($P<0{\cdot}01$)

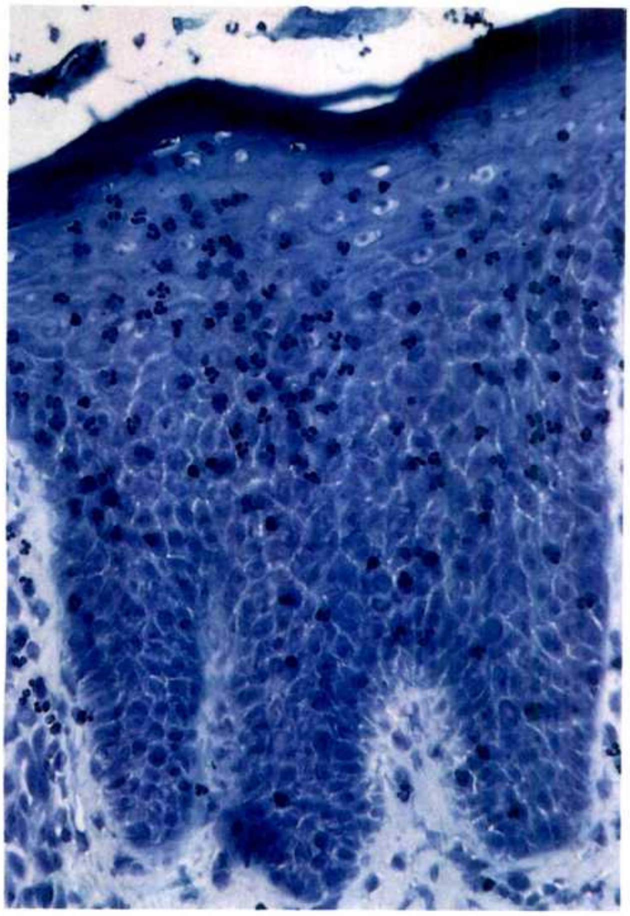

Figure 6. Photomicrograph of neutrophil adherence to psoriasis (×175). In psoriasis neutrophils are observed adherent to epidermis and dermal papillary vascular endothelium.

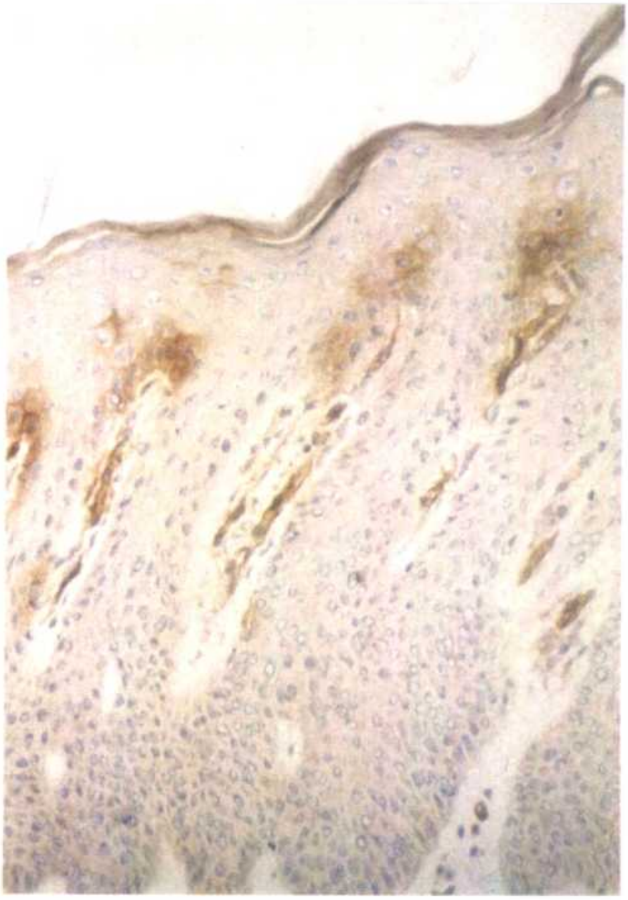

Figure 7. Photomicrograph of psoriasis immunostained for ICAM-1. There is enhanced expression on vascular endothelium and keratinocyte expression at the tips of the dermal papillae (×100).

to adhere to lesional psoriatic epidermis compared with controls (lesional tissue mean 130, SEM ± 31, normal skin mean 9, SEM ± 3 $P<0{\cdot}01$) (Figs 5 and 6). Neutrophils were distributed throughout the epidermis and, although less marked than with lymphocytes, increased adhesion to epidermis overlying papillary tips was observed.

Neutrophils were also found to be adherent to dermal endothelium, and over dermal inflammatory cell aggregates.

Distribution of ICAM-1 in psoriasis

Keratinocyte ICAM-1 expression was observed in all biopsies of psoriasis examined ($n=6$). Expression was focal and confined to the tips of the dermal papillae (Fig. 7). Keratinocytes at the bases of the rete ridges did not express ICAM-1, nor did keratinocytes in normal skin. Endothelial cell expression was observed in normal skin but was more intense in psoriasis, particularly on vascular endothelium in dermal papillae (Fig. 7).

Discussion

Using a frozen-section adhesion assay, we have demonstrated that T lymphocytes and neutrophils preferentially adhere to psoriatic epidermis compared with normal skin, thus indicating an active role for psoriatic epidermis in leucocyte accumulation. In addition, we observed T-lymphocyte adherence to dermal vascular endothelium in psoriasis consistent with previous reports.[5] Epidermal leucocyte adhesion was only observed at 37°C and not at 7°C, consistent with temperature-dependent integrin-mediated adhesion (see below).

In vitro studies demonstrate that lymphocytes adhere to monolayers of human keratinocytes when stimulated by the cytokines IFN-γ or TNF-α.[6,12] Under these experimental conditions, binding activity is inhibited by pretreatment of lymphocytes with monoclonal antibodies to LFA-1 and by pretreatment of keratinocytes with antibodies to the LFA-1 ligand ICAM-1, induced by IFN-γ or TNF-α on the surface of keratinocytes.[6] Short-term organ culture experiments in which whole biopsies of normal human skin are incubated with IFN-γ demonstrate induction of keratinocyte ICAM-1 expression which mediates binding to lymphocytes via LFA-1.[10] Furthermore, immunohistochemical studies demonstrate that IFN-γ *in vivo* induces keratinocyte ICAM-1 expression[13] and that in psoriasis keratinocytes aberrantly express ICAM-1.[7,8] These data suggest that a major receptor/ligand adhesion pathway in lymphocyte adherence to psoriatic epidermis may be LFA-1/ICAM-1.

However, as demonstrated in this study, keratinocyte ICAM-1 expression in psoriasis is focal and confined to epidermis overlying dermal papillae tips, and although maximal adhesion of lymphocytes was observed at this site, lymphocytes were observed in other areas of the epidermis, consistent with immunohistochemical studies showing T cells throughout the epidermis.[14] Furthermore, preliminary studies indicate that antibodies to ICAM-1 and LFA-1 incompletely inhibit lymphocyte adhesion to the epidermis (J.N.W.N.B. *et al.*, unpublished observation), while *in vitro* studies have suggested LFA-1-independent pathways of adherence.[15] Immunohistochemically, keratinocytes have been shown to express several β1-integrins (very late activation (VLA) family) and lymphocyte function associated antigen-3 (LFA-3),[16] and each may potentially mediate lymphocyte adherence. Our results, moreover, are consistent with integrin-mediated adhesion in that adhesion was (a) temperature-dependent, (b) Ca^{2+}/Mg^{2+}-dependent and (c) dependent upon lymphocyte activation. It is of interest that in contrast with T cells, B cells, which also express LFA-1 did not bind in our assay system. The selective presence of T lymphocytes in the epidermis is a feature of psoriasis and other inflammatory skin diseases such as dermatitis and lichen planus. Furthermore, cutaneous T-cell lymphomas display epidermotropism, whereas B-cell lymphomas do not.[17]

Several factors suggest a key role for T lymphocytes in psoriasis including (a) association with certain MHC genes,[18] (b) early accumulation of T cells in evolving psoriatic plaques[14] and, (c) rapid response of psoriasis to cyclosporin A,[19] an agent which specifically inhibits T-cell activation. Furthermore, activated T-cell-derived cytokines induce keratinocyte elaboration of autocrine growth factors[20] and T-cell clones derived from psoriasis induce keratinocyte proliferation *in vitro*.[21]

Neutrophils within the epidermis are also a prominent histological feature of psoriasis. Although their pathogenetic role is unknown, psoriatic epidermis is rich in neutrophil chemoattractants including arachidonic acid metabolites,[22] and cytokines including interleukin 8,[23] suggesting that they are central to the pathophysiology of the disease. Agents targeted at inhibiting leucocyte adherence to the epidermis are likely to prove of therapeutic benefit in psoriasis and other inflammatory cutaneous disorders.

Acknowledgments

J.N.W.N.B. was supported by the Wellcome Trust and the Psoriasis Association and D.M.M. by the Dunhill Medical Trust.

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Family history, smoking habits, alcohol consumption and risk of psoriasis

L.NALDI,* F.PARAZZINI,† A.BREVI,* A.PESERICO,‡ C.VELLER FORNASA,‡ G.GROSSO,‡ E.ROSSI,§ P.MARINARO,§ M.M.POLENGHI,¶ A.FINZI,¶ G.GALBIATI,§ G.RECCHIA,** M.CRISTOFOLINI,** D.SCHENA†† AND T.CAINELLI*

* *Cattedra di Clinica Dermosifilopatica, Università degli Studi di Milano, Ospedali Riuniti di Bergamo, Italy*
† *Istituto di Ricerche Farmacologiche 'Mario Negri', Milano, Italy*
‡ *Clinica Dermatologica, Università di Padova, Italy*
§ *Divisione di Dermatologia, Ospedale di Monza (MI), Italy*
¶ *2 Clinica Dermatologica, Università degli Studi di Milano, Italy*
** *Divisione di Dermatologia, Ospedale Civile di Trento, Italy*
†† *Clinica Dermatologica, Università di Verona, Italy*

Accepted for publication 28 April 1992

Summary

We have conducted a multicentre case-control study to assess the epidemiological importance of previously suggested risk factors for psoriasis, including family history of the disease, smoking and alcohol consumption. Newly diagnosed psoriatics, with a history of skin manifestations no longer than 2 years were eligible as cases; as controls we selected subjects with newly diagnosed dermatological conditions other than psoriasis. Interviews were performed by trained medical investigators using a structured questionnaire. Two-hundred and fifteen cases, aged 16–65 years (median age 38), and 267 controls, aged 15–65 years (median age 36), were interviewed and included in the analysis. Family history was a risk factor for psoriasis; the multiple logistic regression (MLR) adjusted-odds ratio was 18·8 (95% confidence interval 6·4–54·8) for a history in parents, and 3·2 (95% confidence interval 1·5–6·6) for a history in siblings. The risk of psoriasis was higher for current smokers than for those who had never smoked. The MLR adjusted odds ratio was 2·1 (95% confidence interval 1·1–4·0) for people smoking 15 cigarettes or more per day. The risk of psoriasis was higher for alcohol drinkers: compared with teetotallers the MLR adjusted-odds ratios were 1·3 (95% confidence interval 0·8–2·3) for subjects drinking one or two drinks/day and 1·6 (95% confidence interval 0·9 to 3·0) for those drinking three or more. However, the trend in risk was not statistically significant. Our study confirms the role of family history in psoriasis and provides some evidence of a dose–response relationship for an association between smoking habits and psoriasis.

Psoriasis is a common disease characterized by erythematous, scaling plaques scattered over the body, and a chronic course. Genetic and environmental factors have been suggested as playing an aetiological role in the disease.[1,2] Studies of familial predisposition,[3] and the association of psoriasis with different HLA antigens,[4] have indicated the role of genetic factors. Other proposed aetiological factors include chronic trauma to the skin,[5] infections, drugs,[6,7] alcohol consumption and smoking habits.[8-10] However, little effort has been invested in epidemiological studies to clarify the role of each factor in terms of magnitude of relative risk. This may have important implications for a preventive approach to the disease and its consequences (i.e. disability prevention).[11] In view of these uncertainties, we have conducted a case-control study in northern Italy to assess the importance of several risk factors for psoriasis.

Subjects and methods

This case-control study was conducted in four teaching hospitals and two general hospitals in northern Italy. Between January 1988 and August 1990 trained interviewers identified and questioned patients with psoriasis, and controls, using a structured questionnaire.

Correspondence: Dr Luigi Naldi, Cattedra di Clinica Dermosifilopatica, Università degli Studi di Milano, Ospedali Riuniti, L.go Barozzi, 1, 24100 Bergamo, Italy.

This work was conducted within the framework of the Gruppo Italiano Studi Epidemiologici in Dermatologia (GISED).

Oral informed consent was obtained from each subject before the data were collected, and fewer than 2% of the eligible subjects refused to be interviewed. Interviewers had biomedical training and experience in medical interviewing. Information was obtained about socio-demographic factors, personal characteristics and habits, including alcohol use, coffee consumption and smoking habits. A detailed medical history was also taken. We computed the average consumption of alcohol per day, assuming the following pure alcohol contents for each type of drink: 150 ml of wine = 330 ml of beer = 30 ml of spirits = 10–12 g of pure alcohol.[12] With an enrolment of approximately 200 cases and 200 controls a relative risk of about 1·5 for common exposures in the population (frequency of exposure in the control group of about 30%) at the usual levels of α (0·05) and β (0·20) can be detected.

Entry criteria for cases were as follows: first diagnosis ever of psoriasis, made by a dermatologist; awareness of cutaneous lesions for no longer than 2 years before diagnosis.

A total of 215 cases, aged 16–65 years (median age 38) were interviewed. The psoriasis cases were classified as: psoriasis vulgaris (158; 73·5%), guttate (35; 16·3%), palmoplantar pustular (10; 4·6%), generalized pustular (5; 2·3%), and other varieties, including flexural and erythrodermic psoriasis (7; 3·3%). Controls were patients diagnosed for the first time in their lives by a dermatologist as having dermatological conditions other than psoriasis, and seen in the same out-patient services as the cases. Subjects were not included if they were being seen for diseases associated with liver dysfunction (e.g. porphyria cutanea tarda), for cutaneous signs of hepatic disorders (e.g. icterus), or if they had a history of psoriasis. All eligible controls were recruited on randomly selected days. Inclusion of controls was monitored by the co-ordinating centre in order to maintain a balance between cases and controls according to sex and age in decade categories. A total of 267 controls, aged 15–65 years (median age 36) were interviewed. Of these controls, 85 (31·8%) had been seen for eczema, 39 (14·6%) for skin cancers, 38 (14·2%) for urticaria, 26 (9·7%) for skin infections, nine (3·4%) for lichen planus, six (2·2%) for burns, and 64 (24·0%) for other skin diseases. The distribution of cases and controls according to age and sex is shown in Table 1.

Table 1. Distribution of cases and controls according to age and sex

	Cases (215)		Controls (267)	
	No.	(%)	No.	(%)
Age				
15–29	70	(32·6)	95	(35·6)
30–49	78	(36·3)	93	(34·8)
50–65	67	(31·2)	79	(29·6)
Sex				
Male	119	(55·3)	163	(61·1)
Female	96	(44·7)	104	(38·9)

Analysis of data

For each variable, we computed the odds ratios of psoriasis as estimates of relative risks[13] and the corresponding 95% confidence intervals (if the 95% confidence intervals do not overlap 1·0, the estimate could be considered statistically significant at the level of $P<0{\cdot}05$). The significance of the linear trend in risk was assessed by the Mantel test[14] at the usual level of $P<0{\cdot}05$. No allowance was made for multiple testing.

Initially we computed odds ratios from data stratified by age and sex according to the Mantel–Haenszel (MH) procedure.[15] The odds ratios for the history of psoriasis in siblings were obtained from data stratified by age, sex, and number of siblings. Subsequently, to account for the effects of potential confounding factors, unconditional multiple logistic regression (MLR) analysis with maximum likelihood fitting was used.[16] The regression equations included terms for age, sex, education (or occupational status), marital status, history of psoriasis in parents or siblings, alcohol and coffee consumption, smoking habits and, when indicated, number of siblings.

Results

The distribution of cases and controls according to education, occupation and marital status is presented in Table 2. There were no associations between years of education, occupation or marital status and psoriasis.

Table 3 shows the distribution of cases and controls according to family history of psoriasis. The odds ratio of psoriasis was much greater for subjects who reported a history of the disease in at least one parent (MLR adjusted-odds ratio 18·8; 95% confidence interval 6·4–54·8), and was also high in subjects with a history of psoriasis in siblings (MLR adjusted-odds ratio 3·2; 95% confidence interval 1·5–6·6).

Table 4 shows the distribution of cases and controls according to alcohol and coffee consumption. Cases tended to report alcohol consumption more frequently

Table 2. Odds ratios of psoriasis according to education, occupation and marital status

	Cases	Controls	Odds ratios (95% confidence interval)		
			Crude	MH*	MLR†
Education (years)					
<7	67‡	82	1§	1§	1§
7–11	95	98	1·2 (0·7–1·9)	1·1 (0·6–2·3)	1·1 (0·6–1·9)
⩾12	51	87	0·7 (0·4–1·2)	0·8 (0·4–1·7)	0·7 (0·3–1·3)
χ_1^2 trend			1·74 (*P*=0·19)	1·74 (*P*=0·19)	1·51 (*P*=0·22)
Occupational status					
Manual	98	116	1§	1§	1§
Clerical	89	110	1·0 (0·5–1·9)	1·0 (0·7–1·5)	1·0 (0·7–1·6)
Professional	10	15	0·8 (0·2–3·1)	0·8 (0·3–1·9)	0·7 (0·2–2·0)
χ_1^2 trend			0·23 (*P*=0·64)	0·50 (*P*=0·48)	0·22 (*P*=0·64)
Marital status					
Never married	69	98	1§	1§	1§
Married	146	167	1·2 (0·8–1·9)	1·2 (0·8–2·0)	1·2 (0·7–2·0)

* Mantel–Haenszel estimates adjusted for age and sex.

† Multiple logistic regression including terms for age, sex, years of education (or occupation), marital status, history of psoriasis in parents and in siblings, alcohol consumption, coffee consumption and number of cigarettes smoked per day.

‡ Some values missing.

§ Reference category.

than controls; the MH odds ratios of alcohol consumption were 1·4 (95% confidence interval 0·9–2·4) for 1–2 drinks per day and 1·8 (95% confidence intervals 1·1–3·5) for three drinks or more per day; MLR adjusted-odds ratios were consistent with previous estimates, but the trend in risk failed to reach conventional levels of statistical significance. We found no association between coffee consumption and psoriasis.

Table 5 shows the distribution of cases and controls according to smoking habits. Compared with people who had never smoked, MLR adjusted-odds ratio for subjects smoking fewer than 15 cigarettes per day was 1·1 (95%

Table 3. Odds ratio of psoriasis according to family history of psoriasis

	Cases	Controls	Odds ratios (95% confidence interval)		
			Crude	MH*	MLR†
History of psoriasis in parents					
No	171‡	263	1§	1§	1§
Yes	42	4	16·1 (7·0–37·0)	19·0 (7·7–46·4)	18·8 (6·4–54·8)
History of psoriasis in siblings					
No	180	253	1§	1§	1§
Yes	33	14	3·3 (1·7–6·4)	3·8 (1·9–7·5)	3·2 (1·5–6·6)

* Mantel–Haenszel estimates adjusted for age and sex (history in parents), and for age, sex and number of brothers and sisters (history in siblings)

† Multiple logistic regression including terms for age, sex, education, marital status, alcohol consumption, coffee consumption, number of cigarettes smoked per day, and number of siblings.

‡ Some values missing.

§ Reference category.

Table 4. Odds ratio of psoriasis according to alcohol and coffee consumption

	Cases	Controls	Odds ratios (95% confidence interval)		
			Crude	MH*	MLR†
Alcohol consumption					
Non-drinkers	52‡	107	1§	1§	1§
Ex-drinkers	6	11	1·1 (0·0–66·8)	1·0 (0·4–3·2)	1·0 (0·3–3·2)
1–2 drinks/day	61	78	1·6 (0·9–2·6)	1·4 (0·9–2·4)	1·3 (0·8–2·3)
⩾3 drinks/day	83	65	2·6 (1·6–4·2)	1·8 (1·1–3·5)	1·6 (0·9–3·0)
χ_1^2 trend¶			16·96 (P<0·001)	6·45 (P=0·01)	1·89 (P=0·17)
Coffee consumption					
Never/occasionally	30	47	1§	1§	1§
1–2 coffees/day	97	119	1·3 (0·7–2·4)	1·2 (0·6–2·4)	1·1 (0·6–2·0)
⩾3 coffees/day	86	101	1·3 (0·7–2·5)	1·3 (0·7–2·5)	1·2 (0·5–1·8)
χ_1^2 trend			0·87 (P=0·55)	0·87 (P=0·55)	0·04 (P=0·84)

* Mantel–Haenszel estimates adjusted for age and sex.

† Multiple logistic regression including terms for age, sex, education, marital status, history of psoriasis in parents and siblings, number of cigarettes smoked per day, coffee and alcohol consumption.

‡ Some values missing.

§ Reference category.

¶ Ex-drinkers excluded.

Table 5. Odds ratio of psoriasis according to smoking habits

	Cases	Controls	Odds ratios (95% confidence interval)		
			Crude	MH*	MLR†
Cigarettes per day					
Never smokers	79‡	133	1§	1§	1§
Ex-smokers	33	38	1·5 (0·8–2·7)	1·5 (0·7–2·9)	1·1 (0·6–2·0)
Current smokers:					
<15 cigarettes/day	54	66	1·4 (0·8–2·3)	1·2 (0·7–2·0)	1·1 (0·6–1·8)
⩾15 cigarettes/day	47	30	2·6 (1·5–4·6)	2·0 (1·2–3·4)	2·1 (1·1–4·0)
χ_1^2 trend¶			12·45 (P<0·001)	12·45 (P<0·001)	4·60 (P=0·03)
Duration of smoking (years)					
Never smokers	79	133	1§	1§	1§
<15 years	67	72	1·6 (0·9–2·5)	1·5 (0·9–2·5)	1·5 (0·9–2·4)
⩾15 years	68	62	1·8 (1·2–2·9)	1·5 (0·9–2·6)	1·2 (0·7–2·1)
χ_1^2 trend			8·0 (P=0·005)	3·64 (P=0·06)	0·47 (P=0·49)

* Mantel–Haenszel estimates adjusted for age and sex.

† Multiple logistic regression including terms for age, sex, education, marital status, history of psoriasis in parents and siblings, alcohol and coffee consumption.

‡ Some values missing.

§ Reference category.

¶ Ex-smokers excluded.

confidence interval 0·6–1·8) and for those smoking 15 cigarettes or more it was 2·1 (95% confidence interval 1·1–4·0). The trend in risk was statistically significant (χ_1^2 trend 4·6, P=0·03). There was no clear relationship between duration of smoking and risk of psoriasis.

Discussion

This study confirms the importance of family history in psoriasis and provides quantitative estimates of the role of smoking habits as a risk factor for psoriasis. The risk of psoriasis was also high in alcohol drinkers, but this was not statistically significant after allowing for potential confounding factors (including smoking) in the multivariate analysis. There was no relationship between education, occupation, marital status or coffee consumption and risk of the disease.

Before discussing the results, potential biases should be briefly considered. Recall bias is probably the most important bias in the study of family history of psoriasis, because cases may be more aware than controls of their family histories. However, in our study we investigated the family history of psoriasis in first-degree relatives only. The assessment of alcohol consumption was based on self-reporting and we recognize that appreciable underestimating can occur.[17] In Italy, however, moderate alcohol consumption by both men and women is socially accepted.[18,19] and the potential association between alcohol, smoking and psoriasis has never received widespread attention in the population. The interviewers were not blind to the status of subjects as cases or controls. However, they were not aware of the potential of the factors investigated, i.e. smoking habits and alcohol consumption, as risk factors of psoriasis. Moreover, questions on alcohol consumption and smoking habits were included in a questionaire recording several other pieces of information, so that the attention of the interviewed subjects and of the interviewers was not focused on the questions in which we were particularly interested. It is reassuring,[20] for example, that in our study no association emerged for coffee consumption, an exposure frequently reported as a risk factor for disease in humans. We included in our study only cases and controls newly diagnosed by dermatologists, in order to limit the assessment to the exposures that preceded the relevant diagnosis. Dermatological controls were selected to minimize the variables connected with the determinants of seeking medical observation (e.g. degree of attention to health problems) in the comparison between cases and controls. Cases and controls were drawn from institutions covering comparable catchment areas, and the rate of participation was almost complete. The multivariate models were designed to indicate and quantify the effects of known or potential confounding factors. Given our results, it is unlikely that confounding factors accounted for the results observed, as multivariate analysis did not appreciably modify any of the estimated relative risks, with the exception of alcohol consumption. That there is a familial predisposition for psoriasis has been recognized for a long time,[1-3] but the reported frequency of a family history in psoriasis varies from 4 to 91%.[3] Most of this variation probably results from the selection of patients and from the methods employed to evaluate family history (clinical evaluation of relatives vs. anamnestic data). The mode of inheritance of psoriasis is uncertain, and is probably multifactorial and polygenic. Various types of evidence support this hypothesis, including those from the studies of segregation ratio (sib-analysis) of affected and unaffected siblings[21] and the analysis of concordance rates among monozygotic and dizygotic twins.[22] The 'familial predisposition' could, of course, also include familial exposure to environmental factors.

The role of alcohol consumption in psoriasis has received considerable attention in the last decade. Chaput *et al.*[8] showed, in a study of prevalence of psoriasis according to consumption of alcohol and liver diseases, that psoriasis was more common among subjects who drank over 50 g/day of alcohol. In a health interview survey conducted in Norway in 1985, Braathen *et al.*[23] found that men with psoriasis drank more frequently than non-psoriatic men. Monk and Neill,[9] in a study of 100 patients with chronic plaque psoriasis, showed that severe psoriasis was frequently associated with heavy drinking. None of these studies, however, was able to rule out the possibility that alcohol consumption was an epiphenomenon related to the chronic course of the disease. Indirect evidence of a possible role of alcohol in psoriasis was obtained in a cohort study of 1380 patients with psoriasis conducted by Stern *et al.*,[24] who observed that cirrhosis was responsible for more deaths among psoriatic patients than expected in the general population. We are aware of only one previous case-control study concerning the relation of alcohol to psoriasis. In a study (limited to males) of 144 cases of psoriasis and 285 controls, Poikolainen *et al.* showed an association between alcohol intake and psoriasis (odds ratio of ever drinkers with respect to teetotallers 2·2), but they suggested that epidemiological studies on incident cases were needed to confirm their association.[10] We have included in our study only cases newly diagnosed by a dermatologist, and aware of their skin lesions for no longer than 2 years, reducing the possibility that the increased alcohol consumption might be secondary to the disability produced by the psoriasis. In our study, the risk of psoriasis increased with the number of drinks. However, this was not significant after taking into account potential covariates, chiefly smoking.

Some evidence concerning the relationship of smoking to psoriasis has been accumulated in recent years. In 1985, Kavli *et al.*[25] reported an increased prevalence of psoriasis among male smokers, but the risk of the disease decreased as the number of cigarettes smoked increased. Braathen *et al.*[23] reported a higher prevalence of smoking among psoriatics than non-psoriatics, and the association was especially strong in women. Indirect evidence for a possible role of smoking habits in psoriasis emerged from a study conducted by Lindegård on a cohort of 159,200 native Swedes.[26] There was an excess of deaths caused by diseases traditionally associated with smoking, such as lung cancer and myocardial infarction, among women with psoriasis. In 1985, O'Doherty and MacIntyre conducted a case-control study of 216 patients with palmoplantar pustulosis, a condition considered by most investigators to be related to psoriasis, and 626 dermatological controls.[27] There was a high risk of the disease in smokers (odds ratio 7·2). Our study has shown an association between psoriasis and smoking habits, independent of alcohol consumption. Only tentative biological interpretations of the role of smoking habits in psoriasis are possible. Recent studies have shown that smoking induces functional and morphological alterations of polymorphonuclear leucocytes, and some investigators have suggested that smoking may cause an exaggerated release of chemotactic factors.[28-30] Smoking may be an indicator of a life-style which includes 'stress', and future research analysing the role of environmental factors should, in our opinion, also consider this factor.

Acknowledgments

The generous contributions of the following Pharmaceutical Companies are gratefully acknowledged: Cilag, Janssen, Roche, Schering. We wish to thank Professor Carlo La Vecchia for his useful suggestions in the preparation of the manuscript, Dr Barbara D'Avanzo for assistance in data analysis, and all dermatologists in the participating centres for making this study feasible.

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British Journal of Dermatology (1992) 127, 218–220.

No evidence for a spirochaetal origin of localized scleroderma

G.RAGUIN, S.BOISNIC,* P.SOUTEYRAND,† G.BARANTON,‡ J.C.PIETTE, P.GODEAU AND C.FRANCÈS§

Department of Internal Medicine, Hôpital Pitié-Salpêtrière, Paris, France
* *Department of Pathology, Hôpital Pitié-Salpêtrière, Paris, France*
† *Department of Dermatology, Hotel Dieu, Clermont-Ferrand, France*
‡ *Unit of Molecular and Medical Bacteriology, Institut Pasteur, Paris, France*
§ *Unit of Dermatology, Hôpital Pitié-Salpêtrière, Paris, France*

Accepted for publication 14 April 1992

Summary We looked for evidence of a *Borrelia* infection in 15 patients with morphoea. We were not able to detect antibodies to *Borrelia burgdorferi* in any of these 15 patients. None of the 14 skin biopsies examined by immunohistochemistry showed evidence of spirochaetes. Skin biopsies were cultured in 10 patients. All were negative. These results do not support a spirochaetal origin of localized scleroderma.

The recognized forms of cutaneous Lyme disease are erythema chronicum migrans, acrodermatitis chronica atrophicans (ACA), and lymphadenosis benigna cutis.[1,2] Localized scleroderma (morphoea) is a skin disease of unknown aetiology which shares some clinical and histopathological features with ACA. Aberer *et al.*[3–5] suggested that localized scleroderma might also be a manifestation of *Borrelia burgdorferi* infection. We have looked for evidence of a *Borrelia* infection in 15 patients with morphoea by serologial, immunohistochemical and microbiological means.

Methods

Patients

Fifteen patients with morphoea were investigated. The female/male ratio was 6:5. Mean age was 48 years (range 6–69). Patients were questioned about tick bites prior to the onset of skin lesions, the duration of skin lesions, previous antibiotic therapy and accompanying symptoms. Morphoea was diagnosed clinically and confirmed histologically.

Serological tests

Serum samples were examined by both immunofluorescence and enzyme-linked immunosorbent assay (ELISA). The strain used as antigen was a French human isolate (IP1) similar to ATCC 35210 strain.[6] Briefly, the indirect immunofluorescence test was performed with a polyvalent conjugate after preadsorption of the serum by a *Treponema phagedenis* sonicate (Behring, Marburg, Germany). An inverse titre of 256 or more was considered as positive. The immunofluorescence results were confirmed with an ELISA detecting IgG and IgM antibodies. A whole-cell sonicate was used as antigen. Optical densities of 0·300 (IgM) and 0·700 (IgG), determined by examination of 100 normal sera, were considered as cut-off values.

Culture of Borrelia burgdorferi

Skin biopsies from 10 patients were cultured in Barbour–Stoenner–Kelly's medium.[7]

Skin biopsies

Skin biopsies were taken from the lilac ring of the lesion for histological examination, and for culture in Barbour–Stoenner–Kelly's (BSK) medium. Small blocks of tissue samples were frozen directly in isopentane pre-cooled for 20 min in liquid nitrogen and stored, until use, at −80°C. In each case, a small part of the biopsy was fixed in Bouin's solution, paraffin-embedded and processed for conventional light microscopy using haematoxylin and eosin staining.

Immunohistochemical studies

Cryostat sections (4 μm) were fixed for 10 min in cold acetone and then treated to inactivate endogenous peroxidase with a methanol solution containing 0·3%

Correspondence: Dr C Francès, Department of Internal Medicine, Hôpital Pitiè-Salpêtrière, Paris, France

hydrogen peroxide for 20 min. They were then incubated for 45 min with appropriate monoclonal antibodies against *Borrelia burgdorferi*: H5332 anti-OspA, H3TS anti-OspA, H9724 anti-flagellin (dilution 1/20; Symbicom Laboratory, S-901, 24 Umea, Sweden). After each incubation, the slides were rinsed with three changes of phosphate-buffered saline (PBS). The samples were then incubated for 30 min with a biotinylated anti-mouse IgG antiserum (1/200 dilution), followed by a third incubation for 30 min with avidin–biotin peroxidase (10 mg/ml avidin with 3 μg/ml biotinylated peroxidase; Vector Laboratory, Burlingam, CA, U.S.A.). The reaction was revealed by diaminobenzidine (10 mg/ 25 ml PBS) and 0·05% hydrogen peroxide (Sigma, St Louis, MO, U.S.A.) for 10 min and mounted in glycerol. In negative controls the primary antibody was replaced by normal mouse serum. A formalin-fixed culture suspension of *Borrelia burgdorferi* strain served as positive control.

Results

Fifteen patients, originating from various regions of France, and presenting with typical morphoea, were studied. Three patients gave a history of tick bites. The duration of the disease ranged from 1 to 48 months (median 16 months). None of the patients had been treated with antibiotics before biopsy. In all patients histology showed typical features of morphoea, with thickened collagen fibres without hyalinization, and in some cases a sparse dermal or perivascular infiltrate of lymphocytes, histiocytes and plasma cells. Antibodies to *Borrelia burgdorferi* were not detected in any of the patients' sera. No spirochaetes were isolated from any of the 10 skin biopsies cultured, nor were spirochaetes detected by immunohistochemical studies in sections from the skin biopsies of 14 patients.

Discussion

Localized scleroderma is a skin disease of unknown aetiology, characterized by circumscribed sclerotic plaques with an ivory-coloured centre and a surrounding violaceous halo. Several factors, including trauma and infection, have been implicated in the pathogenesis of morphoea. Lyme disease is a multisystem infectious disease caused by *Borrelia burgdorferi*. Erythema chronicum migrans (ECM), acrodermatitis chronica atrophicans (ACA) and lymphadenosis benigna cutis are recognized forms of cutaneous Lyme disease.[1] Recently, Aberer *et al.*[3–5] suggested that localized scleroderma (morphoea) might also be a cutaneous manifestation of Lyme disease, on the basis of serological, immunohistochemical and bacteriological data. Aberer *et al.*[4] studied 15 patients with morphoea, and found antibodies to *B. burgdorferi* in eight. Antibodies were absent in 46 control serum samples. Other authors,[8–12] however, did not find significant specific antibody titres in patients with localized scleroderma, and none of our 15 patients had significant antibody titres. Serological testing may be negative during the first few weeks of the illness but, because the sensitivity of the serological tests increases with the duration of the disease, patients with morphoea should be seropositive if this micro-organism was the cause of the disease. Berger *et al.*[2] examined 11 skin biopsy specimens from eight patients with localized scleroderma. No spirochaetes were detected on Warthin–Starry staining. In the study of Aberer *et al.*,[4] spirochaetes were demonstrated in three of eight skin biopsies by an avidin–biotin immunoperoxidase method. These authors used as an antibody the serum of a patient with a high antibody titre against borrelia for detection of *B. burgdorferi* in skin biopsies. Detection of specific antigen with monoclonal antibodies might be a more convenient method for demonstration of *B. burgdorferi* in the skin. We examined 14 skin biopsy specimens from 15 patients with morphoea by an avidin–biotin immunoperoxidase method, using monoclonal antibodies specific for major surface protein OspA and the predominant flagellar protein of *B. burgdorferi* (H9724). Monoclonal antibody H9724 is specific for a flagellar epitope of the genus *Borrelia*.[13] Representatives of the spirochaetal genera *Treponema*, *Spirochaeta* and *Leptospira* do not have a similar antigen. The major surface protein OspA is common to all spirochaetes, but isolates of *B. burgdorferi* are heterogeneous in the types of OspA proteins represented. Monoclonal antibody H5332 recognizes the majority of *B. burgdorferi* isolates.[14] It does not bind to representative borreliae, treponemes and leptospires. H3TS recognizes only some of the European isolates.[14] None of the skin samples reacted with any of the three specific monoclonal antibodies used in our study. These results do not eliminate the possibility of strains non-reactive to the three monoclonal antibodies used in this study. However, such a situation would be most unlikely. More importantly, Aberer *et al.*[4] were able to isolate spirochaetes from one skin biopsy, and these resembled *Borrelia burgdorferi* in motility and shape. The organisms reacted with a polyclonal antiserum, but were not studied with monoclonal antibodies. Isolation and cultivation of spirochaetes from lesions is difficult, and in our study attempts to cultivate *B. burgdorferi* from 10 skin biopsy samples were unsuccessful.

In conclusion, the negative serology, and the absence of spirochaetes in culture and in histological sections, do not support an aetiological role for *B. burgdorferi* in morphoea, at least in our patients. However, we cannot completely exclude the possibility that some cases of localized scleroderma might be a manifestation of *B. burgdorferi* infection. Localized scleroderma might represent a reaction pattern with various aetiologies. The agent of Lyme disease might be responsible for some cases, although this has not yet been proved by appropriate means. Additional studies, with a large number of patients from a recognized endemic area, should be performed, using, as we did in our study, immunohistochemical methods with appropriate monoclonal antibodies, and cultivation of skin biopsies.

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British Journal of Dermatology (1992) **127**, 221–227.

Disturbed extruding mechanism of lamellar bodies in dry non-eczematous skin of atopics

M.FARTASCH, I.D.BASSUKAS AND T.L.DIEPGEN
Department of Dermatology, University of Erlangen, Germany

Accepted for publication 30 March 1992

Summary A characteristic feature of non-eczematous atopic dry skin (DS) appears to be an impaired water permeability barrier (WPB) function. The WPB is constituted by intercellular lipid lamellae, located between the horny cells of the stratum corneum (SC), which are provided by exocytosis of lamellar bodies (LB). The aim of this study was to elucidate whether alterations in the dynamics of LB-extrusion could be responsible for this WPB disturbance. In an ultrastructural morphometric comparison the relative volume of LB in the two uppermost subcorneal layers in DS of atopics ($n=9$) and healthy skin of controls ($n=7$) was determined. The LBs were differentiated into extracytoplasmic LB, i.e. with the cell membrane already fused, and intracytoplasmic LB, i.e. entirely located within the cell. The total volume in the two cell layers of the stratum granulosum did not differ between atopics and controls. However, separate evaluation of the two LB-compartments revealed statistically significant differences between atopics and healthy controls. In the second uppermost cell layer of the stratum granulosum only 13% of the total LB volume of this layer had already fused with the cell membrane in the atopics as opposed to 42% in the controls. On the other hand more LB remained undelivered within the cells of the uppermost SG cell layer of the atopics (26% in atopics versus 8% in controls, $P<0{\cdot}01$). These findings suggest that a pathological extruding mechanism of LB in DS may be at least partly responsible for the recently detected biochemical alterations of epidermal lipids, and for the deficient WPB.

Changes of biophysical properties of the stratum corneum, especially of the water-permeability barrier (WPB) and water-holding function, appear to be characteristic features of the dry non-eczematous skin (DS) of patients with atopic eczema (AE).[1–9] The term 'dry skin' refers to the appearance of the superficial part of the stratum corneum (SC).[10] Several studies have shown that the DS in atopics shows enhanced transepidermal water loss (TEWL) as a direct reflection of an impaired barrier function of the skin.[2,7–9] The crucial factor in the WPB is the intercellular lipid compartment of the SC.[11] It is composed of approximately equal quantities of ceramides, cholesterol, and free fatty acids, as well as lesser amounts of non-polar lipids and cholesterol sulphate.[12–17] Because of their intercellular localization and their distinctive composition, SC lipids are presumably important not only for the formation of a barrier to transcutaneous water transfer, but probably also control desquamation and cohesiveness of corneocytes[18,19] and the water-holding function of SC.[20] SC lipids originate largely from polar lipid precursors (phospholipids and glycosphingolipids) presumably provided by the cells of the stratum granulosum via exocytosis of the lamellar bodies (LB, synonyms: lamellar granules, membrane-coating granules, Odland bodies, keratinosomes). The epidermal LB first appear in the upper stratum spinosum cells as small membrane-bound organelles with their lipid contents arranged in a lamellar pattern. In the stratum granulosum they eventually cluster along, and fuse with, the apical cell membrane and extrude their contents.

Several lines of evidence indicate that the process of lipid translocation and transformation is disturbed in DS. Furthermore, a comparison of the relative volume of LB in DS of atopic and non-atopic individuals shows a higher relative volume of LB at the level of the granular/cornified layer interface, a finding which has been interpreted as a disturbed maturation of LB.[21] Recent studies suggest that the non-eczematous skin shows abnormalities in epidermal lipid metabolism,[22] and an absolute decrease of total stratum corneum lipids, especially their ceramide content.[9,23,24]

The present study is directed towards elucidating the

Correspondence: Priv.-Doz. Dr med M.Fartasch, Department of Dermatology, University of Erlangen, Hartmannstr. 14, 8520 Erlangen, Germany.

role of LB, particularly concerning the dynamics of lipid extrusion via LB exocytosis, in the disturbed WPB of dry skin in atopic eczema, on the basis of physiological and morphometric ultrastructural studies.

Methods

Subjects

Nine adult patients with either a past or present history of AE (five male, four female), and seven non-atopic control subjects (four male, three female) agreed to participate in the study. Both groups had a similar age distribution (22–32 years). In all patients the diagnosis of AE was made according to the criteria of Hanifin and Rajka[25] and Diepgen *et al.*[5] Further requirements for inclusion in the study were a history of persistent dry skin in non-lesional regions, especially in eczema-free periods, and the absence of eczematous lesions on the lateral aspect of the buttock (site of biopsy). Five patients were free of eczema at the time of biopsy. The affected body surface in the other four patients ranged from 10 to 22% (chronic flexural eczema), and they were not in an active phase of their disease. None of the patients had received systemic treatment for at least 6 months prior to the study. The biopsy site had not been treated with topical medications or emollients for the preceding 2 weeks. The biopsy sites of the atopic patients were graded for dryness according to a method described by Bissett and McBride:[26] grade 0, skin smooth, no detectable dryness; grade 1, skin lustrous, slight ashing visible; grade 2, skin dry, without lustre, moderate ashing covering general surface area; grade 3, skin slightly rough overall with slight scaling, high amounts of ashing covering total area; grade 4, skin has moderate to high roughness, moderate scales with some small cracks, high ashing overall; grade 5, skin highly rough with high scaling, large cracks and high overall ashing.

Transepidermal water loss measurements

The measurements of TEWL were performed at the biopsy sites before punch biopsy, using an Evaporimeter EP1 (Servomed, Stockholm, Sweden) under standardized conditions[27] at a constant room temperature of 21°C and a relative humidity of $47 \pm 5\%$.

Morphological preparations and morphometric analysis

Punch biopsies (4 mm diameter) were taken from buttock skin under local anaesthesia with 1% lidocaine. The specimens were processed for electron microscopy. The tissue samples were minced into small pieces (<1 mm). After fixation with 3% glutaraldehyde according to Peracchia and Mittler,[28] post-fixation in 1% osmium tetroxide in 0·1 M cacodylate buffer, and dehydration in graded alcohol, the specimens were embedded in Epon 812. In addition, tissue samples from the atopic group were also fixed with acrolein vapour (Serra, BRD, Kawai *et al.*[29]) for 2 h, rinsed in buffer for 3 h, and post-fixed with 0·5% ruthenium tetroxide (RuO_4) and 0·25% aqueous potassium ferrocyanide in darkness at 4°C for 1 h.[30] The ultrastructural findings were compared with RuO_4-fixed tissue from healthy skin. Semithin sections (1–2 μm) were stained with 1% methylene blue and examined by light microscopy. Ultrathin sections were stained with ethanolic uranyl acetate plus lead citrate. Electron microscopy was performed by Jeol 100 CX. The relative volume of LB in the two uppermost subcorneal cell layers of the epidermis (two uppermost stratum granulosum cell layers) was measured by applying standard ultrastructural stereological methods.[31,32] Two tissue blocks from each subject were sectioned perpendicular to the skin surface. In randomly selected areas of suprapapillar regions of interfollicular epidermis thin sections were investigated. Three ultrathin sections were mounted on every Formvar-coated copper grid. To avoid bias the middle part of the left upper ultrathin section of each grid was studied.

At least five cells of the two uppermost layers of the stratum granulosum were photographed consecutively, in slightly overlapping fields, at a primary magnification of × 6600. The negatives were printed to give a final magnification of × 66,000. A total of 140 micrographs were taken of each layer (280 micrographs per patient or control). By fitting the slightly overlapping electron micrographs together a complete picture of the two layers was obtained (Fig. 1).

The following procedure was used for the identification of the cell layers to be studied: first, the stratum corneum/stratum granulosum boundary was determined on the electron micrographs and outlined by a marker, then the 'uppermost' granular layer cells ($i=1$) were identified as those which showed direct contact with cells of the SC. Finally the 'second uppermost' granular layer cells ($i=2$), i.e. those cells which showed direct apical contact only with the uppermost stratum granulosum cells—defined as previously described—and no direct contact with the lowest cell layer of the stratum corneum, were outlined. LB were identified by their typical lamellar internal structure and their cell membrane-like coating membrane. Those LB which were

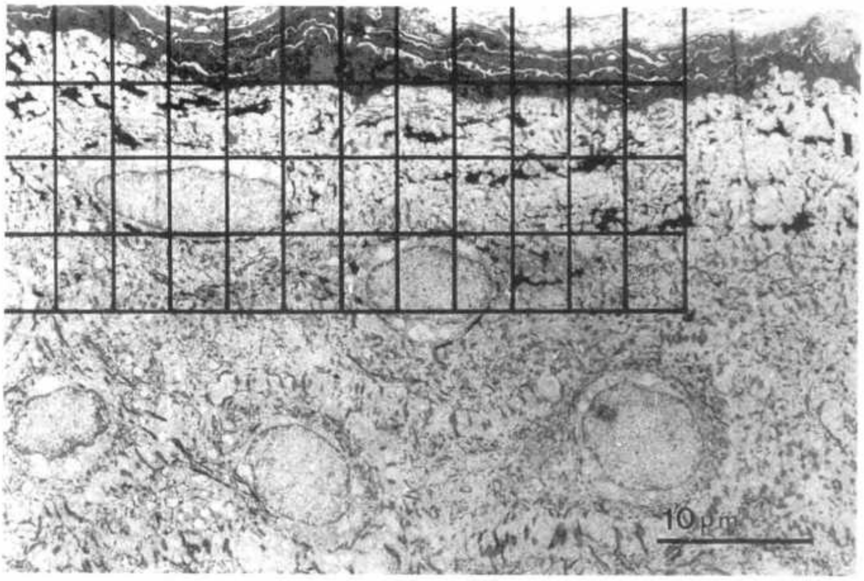

Figure 1. At least five cells of the two uppermost cell layers of the stratum granulosum and the adjacent granular cell/horny cell interface were photographed consecutively (GTA/OsO$_4$ × 2200).

already fused with the cell membrane directly, or indirectly, by building so-called 'LB conglomerates', were defined as 'extracytoplasmic' LB. Those LB which were found in the cytoplasm of the cell were defined as 'intracytoplasmic' LB (Fig. 2, Fig. 3a and b). Intracellular LB conglomerates which could be caused by oblique sectioning were not observed. Using commercial computer software (Videoplan, Kontron, Bildanalyse GmbH, Munich, Germany) the following stereological parameters were measured from sections of at least 5 cells/layer:

% volume of 'extracytoplasmic' LB in layer i

$$= \frac{\text{area of 'extracytoplasmic' LB in layer } i}{\text{total area of cell layer } i} \times 100$$

% volume of 'intracytoplasmic' LB in layer i

$$= \frac{\text{area of 'intracytoplasmic' LB in layer } i}{\text{total area of cell layer } i} \times 100$$

($i = 1$ or 2 for the uppermost or the 'beneath the uppermost' subcorneal cell layers of the epidermis, respectively).

Statistical analyses

Comparisons between patients and controls were made by the Wilcoxon–Mann–Whitney test. All reported P-values are two-tailed.

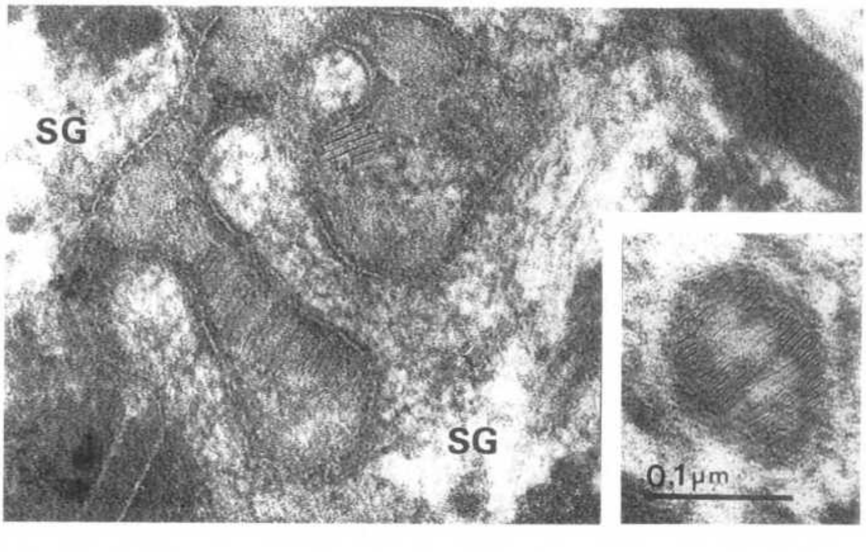

Figure 2. 'Extracytoplasmic' LB which have already fused with the membrane of a stratum granulosum (SG) cell. Inset: 'intracytoplasmic' lamellar body (GTA/OsO$_4$ × 200,000).

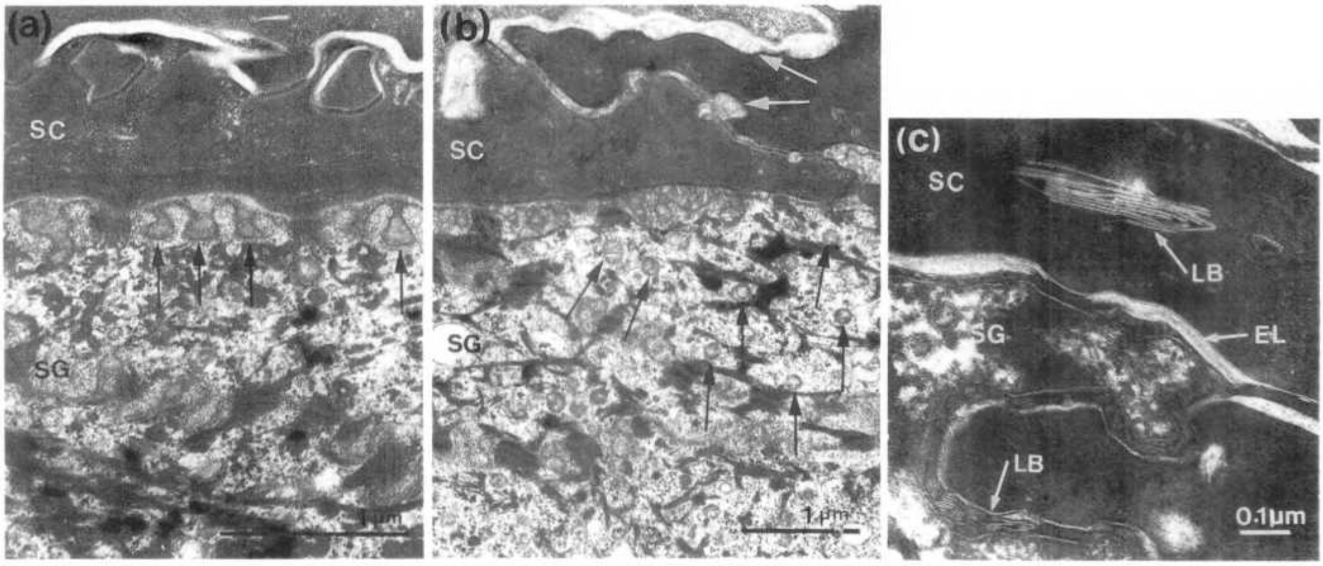

Figure 3. (a) In the upper stratum granulosum (SG) of normal skin the LB (→) cluster and fuse with the apical cell membrane (GTA/OsO_4 × 33,000). (b) In atopic dry skin the LB (black arrows) are found to a great extent still persisting in the cytoplasm of the upper stratum granulosum cell layer. In the intercellular spaces of the stratum corneum (SC) LB-discs are still visible (white arrows) (GTA/OsO_4 × 20,000). (c) Undelivered LB discs are found in the horny cell. Already formed epidermal lipids (EL) and LB discs are found simultaneously in the intercellular space between stratum granulosum (SG) and stratum corneum (SC) (Acrolein/RuO_4 × 90,000).

Results

Skin dryness and TEWL

Of the nine AE patients six showed skin dryness of grade 3, and three of grade 4 at the buttock biopsy sites. Skin dryness grade 0 was registered for all control subjects at the same site. The atopic group showed a significantly higher mean baseline TEWL value (8·6 g/m^2h) than the control group (5·4 g/m^2h) ($P<0{\cdot}01$).

Ultrastructure

In contrast with normal skin where LB-discs disappear regularly one to three cell layers above the stratum granulosum, in the atopic skin LB discs are focally clearly recognized in higher layers (up to five above the stratum granulosum) within the SC interstices. In addition, in some specimens of atopic skin undelivered LB-discs in the cytoplasm of some transit cells, as well as LB-discs in the horny cell matrix, can be found, as shown by modified ruthenium tetroxide staining (Fig. 3c), a finding which is very rare in normal human skin.

Morphometric analyses

The biopsies of atopic skin and of the controls showed no signs of eczema, i.e. parakeratosis, spongiosis, acanthosis and dermal inflammatory cell infiltrate. The thickness of the stratum granulosum as determined on methylene blue-stained semithin sections was 2–3 cell layers in all 16 subjects. Comparing the relative total volumes of LB in controls and atopics, there were no statistically significant differences in the relative volumes of LB in the first uppermost and second uppermost stratum granulosum cell layers (Table 1).

Statistically significant differences were seen in the extruding mechanism of LB. While in the cells 'beneath the uppermost' layer of the stratum granulosum of the healthy skin 42% of the LB had already fused with the apical part of the cell membrane, in the atopic skin only 13% were found adjoining the cell membrane ($P<0{\cdot}05$) (Fig. 4 a and b). The difference was even more obvious in the uppermost layer of the stratum granulosum (Fig. 3a and b). In healthy skin 92% of LB had already fused with the cell membrane as opposed to 74% in the atopic skin ($P<0{\cdot}01$). Thus significantly more non-extruded LB remained undelivered within the uppermost stratum granulosum cells in the atopic skin.

Discussion

Generalized non-eczematous DS is a characteristic and common feature in AE[1,5,7] and appears to be independent of the eczema.[6] In some patients this condition is

Table 1. Morphometric analysis: comparison of relative volumes of the two upper stratum granulosum layers in atopics and controls

Stratum granulosum	Subjects	% volume† extracytoplasmic LB	% volume† intracytoplasmic LB	% volume† of total LBs	%‡ of extracytoplasmic LB
1st cell layer	Atopics ($n=9$)	1·34NS (0·91–2·14)	0·41* (0·13–0·70)	1·75NS (1·07–2·52)	73·7** (66·0–88·1)
	Controls ($n=7$)	1·94 (0·98–3·56)	0·17 (0·04–0·53)	2·11 (1·04–3·61)	91·6 (84·3–98·7)
2nd cell layer	Atopics ($n=9$)	0·08* (0·00–0·66)	0·54NS (0·52–1·58)	0·78NS (0·54–1·58)	12·9* (0·0–57·6)
	Controls ($n=7$)	0·32 (0·14–1·90)	0·57 (0·29–1·36)	1·13 (0·69–2·87)	41·9 (9·3–74·3)

† LB-volume in per cent of total volume of cell layer: median (range).
‡ Extracytoplasmic LB volume in per cent of total LB-volume.
Mann–Whitney *u*-test: * $P<0{\cdot}05$; ** $P<0{\cdot}01$; NS, not significant.

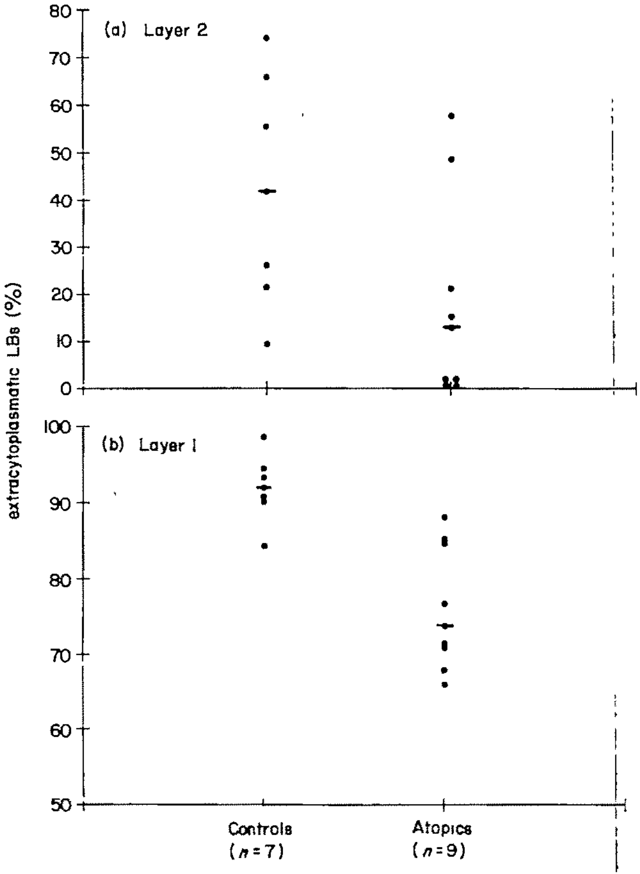

Figure 4. (a) Percentage of relative volume of extracytoplasmic LB in relation to total relative volume of LBs in the second uppermost cell layer of SG (layer 2).(b) Percentage of relative volume of extracytoplasmic LB in relation to total relative volume of LBs in the uppermost layer of SG (layer 1).

lifelong without any additional symptoms. DS is also seen in chronic irritant dermatitis and in normal subjects without AE.[7,10] It is not known whether the functional aspects of barrier function are similar in these conditions.

In the present study the subjects with AE (five of whom had only a past history of AE) had a higher mean base TEWL than the control subjects. This is in agreement with other studies.[2,7-9]

There are conflicting data as to the cause of DS in non-eczematous skin of atopics. In previous studies coexistance of AE with autosomal dominant ichthyosis was suggested as a cause of DS in as many as 30–40% of atopics.[33,34] However, we have previously shown that few atopics have concomitant autosomal dominant ichthyosis (4–6%) and that the dry skin condition in AE is structurally distinguishable from DS in dominant ichthyosis.[35] Moreover, we could not confirm the previously proposed thesis that the persistent dry skin of atopics is the result of subclinical eczema. We did, however, find some evidence of disturbed maturation of WPB in atopic non-eczematous dry skin. Whereas most recent studies of the non-eczematous dry skin of atopics have focused on morphological ultrastructural quantification,[21] light microscopical description of the epidermis[33] or quantitative biochemical analysis of the lipid contents of the DS,[9,22-24] we focused on the dynamics of LB-extrusion in the present study. The separate evaluation of the LB-compartments ('intracellular', and already fused 'extracellular' LB) showed that almost

26% of total LB volume in atopics (vs. 8% in controls) is still found in the cytoplasm of the upper stratum granulosum cell layer. We could thus visualize both a delayed, and probably incomplete extrusion of LB, resulting in diminished delivery of their 'probarrier' polar lipids in the intercellular space. The fate of the undelivered or retained LB is not clear. Some are probably 'keratinized', others might be processed by catabolic enzymes so that they can no longer be detected by ultrastructural means. LB-discs in the corneocytes of normal skin are rarely encountered. Comparing atopic skin with normal skin, our impression was that these remnants were found more often in the horny matrix of DS-atopic skin, as shown by ruthenium tetroxide fixation. In contrast with previous studies[21] which have been restricted to the LB at the level of the granular/cornified layer interface, we could not find any difference in the relative volume of LB per stratum granulosum cell layer in atopics compared with controls.

Apart from the probable diminution of the supplied polar lipids, the delayed LB exocytosis may additionally impair the formation of the WPB by disturbing other, presumably LB-dependent processes, such as lipid transformation[19] and acidification of the intercellular spaces of the granular/cornified layer interface to the pH optimum of the simultaneously extruded acid hydrolases. Thus the activity of these enzymes might be influenced.[36] In addition, the persistence of LB-derived discs at higher levels within atopic SC interstices implies that LB contents are not processed normally in DS. Such alteration in the orderly conversion of LB-derived discs to lipid lamellae in SC could also explain the abnormal barrier function and/or the pathological desquamation of atopic DS, and has also recently been described in psoriatic SC.[37]

Healthy skin has the ability to regulate disturbed barrier function by increased lipid synthesis (positive feedback regulatory mechanism of TEWL).[38,39] Because of the altered extruding mechanism of LB, which may be at least partly responsible for the deficient WPB and the resulting susceptibility of the atopic skin to irritants and infections, the ability to regulate the barrier function is reduced and this may evoke immunological and inflammatory mediators in the epidermis, finally leading to clinically visible atopic eczema. To date it is not known whether these alterations in LB are also found in other pathological conditions leading to dry skin, but examination of such disorders from the new angle of LB dynamics may provide further insight into the mechanisms involved.

In addition, recent biochemical studies have shown that local UV irradiation of healthy skin leads to an increase of SC ceramides,[40] and it is has been speculated that the dramatic improvement of the impaired WPB, and decrease of irritability in DS of atopics following UV irradiation may be partly related to the enhancement of the diminished SC ceramides, which are functionally important for the stability of the intercellular multilamellar lipid sheets.[40] On the other hand, ultrastructural studies have suggested that UV irradiation has a direct influence on the LB-extruding behaviour,[41] so that the improvement of barrier function following UV irradiation, especially in DS of atopics, might be induced by an increased outward movement of LB into the extracellular space, and an improved conversion of LB-derived discs into lipid lamellae of SC.

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Selective activation of circulating CD4$^+$ lymphocytes in severe adult atopic dermatitis

J.M.SOWDEN, R.J.POWELL* AND B.R.ALLEN

*Department of Dermatology and * Clinical Immunology Unit, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH, U.K*

Accepted for publication 6 May 1992

Summary An analysis of peripheral blood lymphocyte subsets and their expression of activation markers was performed using flow cytometry in 12 adult patients with severe atopic dermatitis, and compared with 14 normal individuals. Repeated measurements were made over an 8-week period during which disease activity was also assessed.

Increased percentages of activated and unactivated CD4$^+$ lymphocytes, and decreased percentages of CD8$^+$ cells were observed in atopic dermatitis. Increasing disease activity was associated with an increase in the proportion of activated and unactivated CD4$^+$ lymphocytes and a fall in the proportion of CD8$^+$ cells. This study demonstrates that in adults with severe atopic dermatitis, increasing disease activity is associated with selective activation of CD4$^+$ lymphocytes and a relative expansion of the CD4$^+$ cell subset.

The predominant cell in the cutaneous infiltrate in atopic dermatitis is the T lymphocyte[1] and the majority of these are CD4$^+$ cells.[2] Studies to assess whether peripheral blood lymphocytes reflect these cutaneous changes have produced conflicting results. Whilst there are reports of normal levels of circulating CD4$^+$ and CD8$^+$ lymphocytes in atopic dermatitis,[3,4] other studies have demonstrated an increase in the ratio of CD4$^+$ to CD8$^+$ cells. This has been attributed to an increase in the percentage CD4$^+$ cell count in some reports[5–7] and to a decrease in the proportion of CD8$^+$ lymphocytes in others.[8–12] Furthermore, some investigators have found the ratio of CD4$^+$ to CD8$^+$ cells to be elevated only in patients with severe or extensive atopic dermatitis.[13,14]

Although the skin-infiltrating CD4$^+$ lymphocytes in atopic dermatitis show high levels of HLA-DR expression,[3] previous studies have not assessed the activation status of circulating lymphocytes.

This study was designed to evaluate activated and unactivated circulating T-lymphocyte subsets in a well-characterized group of adult patients with severe atopic dermatitis over an 8-week period. Using flow cytometry the CD2 (pan T cell), CD4 (helper T cell) and CD8 (cytotoxic T cell) proportions were quantified and the degree of activation was assessed by measuring the cell membrane expression of the interleukin-2 receptor (IL-2R or CD25) and the major histocompatibility complex class II antigen HLA-DR.

Patients and methods

Twelve patients with active, severe, refractory atopic dermatitis were studied over an 8-week period. The diagnosis was established by accepted clinical criteria[15] including a personal or family history of atopy. All the patients had required periods of in-patient therapy or treatment with systemic steroids during the 2 years before the study. All systemic treatments were discontinued 2 weeks prior to the study but patients continued to use their standard topical steroids and emollients. Seven of the patients suffered from asthma and continued with their standard inhaled bronchodilator therapy throughout the study. Assessment of disease activity, and venesection, was performed every 2 weeks. Control data was obtained from 14 age- and sex-matched subjects with no personal or family history of atopy. None of the patients or controls was working night-shifts, and the samples were taken on the same day between 9 and 11 a.m. to minimize the effects of diurnal variation on lymphocyte counts. Each 'run' analysed specimens obtained from three patients and one control.

Correspondence: Dr J.M.Sowden.

Assessment of disease activity

Disease activity was measured by a single observer using two previously described[16] clinical scoring systems to assess both disease activity and the extent of disease. In the first, erythema, purulence, excoriation, cracking, dryness and lichenification were scored on a scale of 0–3 at six sites which were defined prior to the study.[16] This gave a total body 'disease activity' score, with a maximum of 108. The second system was based on the 'rule of nines' used for assessing burn victims. At each defined site the extent of the disease was measured by estimating whether none, a third, two thirds or the whole area was affected. The sum of the values from each site gave an 'extent of disease' score which was an assessment of the total affected body area.

Cell separation

Eight millilitres of anticoagulated (EDTA) peripheral venous blood were mixed with an equal volume of RPMI 1640 medium, layered on to lymphocyte separation medium (Ficoll–Hypaque, specific gravity 1·077, Mycomed, Birmingham, U.K.) and centrifuged at 2000 r.p.m. for 20 min. The lymphocyte-rich layer was removed, washed three times in RPMI and resuspended in 1-ml of RPMI. Aliquots (50 μl) from this cell suspension were immediately incubated with 5 μl of the appropriate monoclonal antibody, gently mixed and then stored for 30 min at 4°C. After staining, 1 ml of PBS with 5% fetal calf serum (FCS) was added to prevent cell clumping. This mixture was centrifuged at 1200 r.p.m. for 5 min. The cells were resuspended in PBS with 5% FCS, centrifuged again and finally resuspended and fixed in 400 μl of PBS with 0·5% paraformaldehyde. The cells were analysed within 48 h.

Immunofluorescence and flow cytometry

Table 1 lists the directly conjugated monoclonal antibodies used in the study. Standard Dako and Becton-Dickinson reagents were used for direct staining of CD2, CD4, CD8, HLA-DR and IL-2R. Double-labelling techniques using the fluorochromes fluorescein isothiocyanate (FITC) and R-phycoerythrin (RPE) allowed the identification of both T-cell subsets and activated T cells (expressing HLA-DR or IL-2R). Negative controls containing FITC and RPE fluorochrome-labelled 'irrelevant' antibodies were used to establish the levels of background autofluorescence and non-specific staining. Lymphocytes from atopic dermatitis patients and control subjects were analysed consecutively.

Flow cytometry was performed on 10,000 cells from each sample with a Becton-Dickinson Fluorescence Activated Cell Sorter (FACScan$^\bullet$) and the results were analysed using a Consort 30 programme. Lymphocytes were gated by adjusting the forward and side-scatter channel thresholds to exclude monocytes, polymorphonuclear leucocytes, platelets and cell debris. The percentage of lymphocytes expressing the CD2, CD4 and CD8 markers was obtained and dual colour analysis gave the percentage of CD2$^+$, CD4$^+$ and CD8$^+$ lymphocytes expressing the HLA-DR or the IL-2R activation markers.

Statistical analysis

Analysis of data was performed using a SPSSX 4.0$^\bullet$ computer package. Comparisons between patients and controls were made using the Mann–Whitney *U*-test. To exclude autocorrelation between the sequential measurements in a given patient over the 8-week period, the Durbin–Watson test was applied. The data were then analysed using Spearman's rank correlation test.

Table 1. Monoclonal antibodies used to define T-cell subsets

Monoclonal antibody (supplier)*	CD specificity	Cell type	Fluorochrome†
T11 (Dako)	CD2	T cells, natural killer cells	FITC
T4 (Dako)	CD4	T helper/inducer	FITC
T8 (Dako)	CD8	T cytotoxic	FITC
HLA-DR (BD)	HLA-DR	Activated T cells, monocytes and B cells	RPE
IL-2R (BD)	CD25	Activated T cells, monocytes and B cells	RPE

* Dako, Dakopatts; BD, Becton-Dickinson.

† FITC, fluoroscein isothiocyanate; RPE, R-Phycoerythrin.

Table 2. Demographic characteristics of patients with atopic dermatitis and controls

	Atopic dermatitis	Controls
Number of patients	12	14
Sex	9M	9M
	3F	5F
Age		
Range (years)	15–48	15–44
Mean (years)	25·8	26·7

Results

The age and sex-distributions between the atopic dermatitis patients and the control subjects were similar (Table 2).

In the patients with atopic dermatitis 'extent of disease' scores over the 8-week study ranged from 15 to 99% (mean 57·4%) and 'disease activity' scores ranged from 10 to 64 (mean 37·0). Eleven of the 12 patients had IgE levels persistently greater than 1000 kU/l throughout the study period and one patient had normal IgE levels (<256 kU/l).

In atopic dermatitis the percentages of CD2$^+$ and CD4$^+$ lymphocytes were elevated and the percentage of CD8$^+$ cells was decreased (Table 3). There was no difference in the proportion of CD2$^+$ CD4$^-$ CD8$^-$ cells between the two groups.

The percentage of CD4$^+$ lymphocytes expressing the activation markers HLA-DR and IL-2R was increased in patients with atopic dermatitis. The percentage counts for HLA-DR$^+$ and IL-2R$^+$ CD8 lymphocytes were within the normal range.

There was a good level of correlation between the two clinical scoring systems used, 'disease activity' and 'extent of disease' (Spearman's rank correlation test, r=0·67, P=0·0001). Consequently, only the results from the analyses using the 'disease activity' scores are presented, as the results from the 'extent of disease' data were comparable.

The Durbin–Watson values for CD4$^+$ (d=1·91, n=73), HLA-DR$^+$ CD4$^+$ (d=2·26, n=73) and IL-2R$^+$ CD4$^+$ (d=2·36, n=73) lymphocyte results showed no autocorrelation between sequential measurements. Spearman's rank correlation test demonstrated that worsening disease was associated with an increase in the percentages of CD4$^+$ (r=0·36, P<0·01), activated CD4$^+$ (HLA-DR$^+$ CD4$^+$ r=0·60, P=0·0001; IL-2R$^+$ CD4$^+$ r=0·48, P=0·001) and unactivated CD4$^+$ lymphocytes (HLA-DR$^-$ CD4$^+$ r=0·30, P<0·05; IL-2R$^-$ CD4$^+$ r=0·31, P<0·05). The association between disease activity and HLA-DR$^+$ CD4$^+$ lymphocytes is shown in Figure 1. Increasing disease activity was associated with a fall in the proportion of CD8$^+$ lymphocytes (r=−0·31, P<0·01) but no significant change in the percentage of activated CD8 cells (HLA-DR$^+$ CD8$^+$ r=−0·15, P=0·2, IL-2R$^+$ CD8$^+$ r=−0·22, P=0·23).

There was a high level of correlation between HLA-DR$^+$ CD4$^+$ and IL-2R$^+$ CD4$^+$ lymphocytes (Spearman's rank correlation test, r=0·73, P=0·0001).

Table 3. Mean (SE) percentage counts for lymphocyte subsets in patients with atopic dermatitis and controls. (Pooled data obtained from 12 patients and 14 controls over an 8-week period.)

	Percentage count (%)		
	Atopic dermatitis n=73	Control n=26	Significance (P)
CD2$^+$	79·05 (0·98)	73·44 (1·67)	<0·005
CD4$^+$	50·42 (1·27)	40·73 (1·13)	<0·0001
CD8$^+$	27·82 (0·90)	32·19 (1·13)	<0·01
HLA-DR$^+$ CD2	9·19 (0·52)	5·27 (0·43)	<0·0001
HLA-DR$^+$ CD4	7·81 (0·58)	3·13 (0·2)	<0·0001
HLA-DR$^+$ CD8	1·71 (0·12)	1·67 (0·18)	NS
IL-2R$^+$ CD2	11·02 (0·52)	6·72 (0·45)	<0·0001
IL-2R$^+$ CD4	9·81 (0·53)	5·88 (0·42)	<0·001
IL-2R$^+$ CD8	1·16 (0·08)	0·96 (0·19)	NS

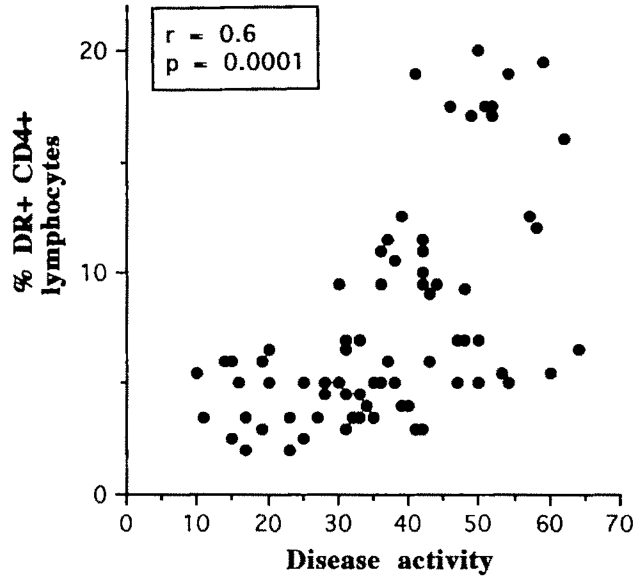

Figure 1. HLA-DR$^+$ CD4$^+$ lymphocyte counts against disease activity. (Pooled data from 12 patients with atopic dermatitis over the 8-week study period.)

Discussion

This study demonstrates that patients with severe adult atopic dermatitis show an increase in the proportion of peripheral blood lymphocytes expressing the CD2 marker; CD2 being expressed by CD4$^+$, CD8$^+$ lymphocytes and natural killer cells. Although natural killer cells were not specifically quantified there appeared to be no increase in the proportion of CD2$^+$ CD4$^-$ CD8$^-$ cells.

There was an increase in the percentage of circulating CD4$^+$ lymphocytes and a decrease in the percentage of CD8$^+$ cells in atopic dermatitis; these changes became increasingly significant with worsening disease. The proportion of activated CD4$^+$ lymphocytes was increased in atopic dermatitis and these changes also became more obvious with worsening disease. There was no increase in the proportion of activated CD8 lymphocytes.

Our results are consistent with the observation that the majority of CD4$^+$ lymphocytes in the cutaneous infiltrate of atopic dermatitis are HLA-DR$^+$ CD4 cells.[2,3] Quantification of T-cell subsets in tissue sections can be problematical, especially when the inflammatory infiltrate is dense,[2] and because the methods lack objectivity and reproducibility, repeated or comparative assessments may be invalid. The study of circulating lymphocyte subpopulations has been encouraged by the knowledge that skin lymphocytes are not a static population, and a constant flow of cells to and from regional lymph nodes occurs via the peripheral blood. Venesection is less traumatic than skin biopsy and allows objective and reproducible analysis of blood lymphocytes using monoclonal antibodies and flow cytometry. Despite these advantages, the results from previous studies of circulating T lymphocytes in atopic dermatitis have produced conflicting results. Some of these differences may have been due to variations in experimental technique, the age of the patients or the activity of the dermatitis. We chose to study adult patients with severe atopic dermatitis because we felt that any changes in the T-cell populations would be most apparent in such a group. Our clinical scoring system was based on that used in other clinical trials[16,17] and was sufficiently sensitive to detect changes in both the activity and the extent of atopic dermatitis over an 8-week period. Before conclusions pertinent to the full spectrum of atopic dermatitis can be made, similar studies will be necessary in patients with milder disease.

Studies of the soluble IL-2 receptor (sIL-2R) have provided further evidence to support the association between lymphocyte activation and disease activity in atopic dermatitis. Increased serum levels of sIL-2R have been correlated with disease activity in atopic dermatitis[18,19] and it is likely that the activation of CD4$^+$ lymphocytes contributed to these increased levels. The expression of IL-2R by activated T cells is associated with cell proliferation.[20] Our study demonstrates not only an increase in the expression of IL-2R by CD4$^+$ lymphocytes but also a proportional increase in the CD4$^+$ cell population, suggesting that selective activation and proliferation of circulating CD4$^+$ lymphocytes occurs in severe atopic dermatitis.

Many of the current treatments for atopic dermatitis modify the immune response. Corticosteroids and PUVA impair the function of antigen-presenting cells[21,22] and inhibit the activation and proliferation of lymphocytes.[23,24] Cyclosporin has a profound inhibitory effect on CD4$^+$ lymphocyte activation and proliferation, but no effects on CD8$^+$ activity,[25] and has been shown to be a highly effective treatment in patients with severe atopic dermatitis.[16,26,27] This lends additional support to the view that cellular immune mechanisms, in particular the selective activation of CD4$^+$ lymphocytes, are important in the pathogenesis of atopic dermatitis.[28,29]

Acknowledgment

We thank Mr J.Ransom for performing the flow cytometry.

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Possible mechanisms of immune modulation in chronic dermatophytoses: an *in vitro* study

J.M.McGREGOR, A.J.HAMILTON AND R.J.HAY
Dunhill Research Laboratories, UMDS (Guy's Campus), St Thomas' Street, London SE1 9RT, U.K.

Accepted for publication 15 April 1992

Summary

It has been suggested that patients with chronic superficial *Trichophyton rubrum* infection have defective cellular immunity to dermatophyte antigens. This may be due to a selective anergy to dermatophyte antigens or reflect the activity of dermatophyte-derived lymphocyte inhibitory factors. To explore these possibilities, we assessed lymphocyte transformation to a variety of recall antigens, including a cytoplasmic and exoantigen preparation of *Trichophyton rubrum* in 15 patients with chronic dermatophyte infection and 15 age- and sex-matched positive controls. In a duplicate set of experiments, autologous serum was replaced by heat-inactivated fetal calf serum. In addition, the direct effect of *Trichophyton rubrum* extracts on lymphoproliferation was assessed *in vitro*.

Comparable lympocyte transformation to each recall antigen was observed in patients and controls. Moreover, we found no evidence for a circulating dermatophyte-derived lymphocyte inhibitory factor in sera from patients with chronic superficial infection. A direct inhibitory effect of *Trichophyton rubrum* on lymphocyte proliferation to recall antigens was observed, however, at protein concentrations of >10 μg/ml (exoantigen preparation) and >25 μg/ml (cytoplasmic preparation). This inhibitory effect was rapidly reversible, not associated with loss of cell viability and maximal when added within 24 h of antigen to cultured lymphocytes. Class II MHC antigen HLA-DR, a surface marker of T-cell activation, was observed on inhibited lymphocytes co-cultured with antigen, suggesting the primary target for the inhibitory effect *in vitro* is the lymphocyte rather than the antigen-presenting cell. These data are consistent with the hypothesis that lack of cutaneous inflammation at the site of *Trichophyton rubrum* infection in the human host may reflect a local effect of dermatophyte-derived lymphocyte inhibitory factors. This contrasts with previous suggestions that patients predisposed to chronic dermatophytoses might exhibit a selective anergy to *Trichophyton rubrum*.

Dermatophytes comprise a group of superficial fungal pathogens exhibiting pronounced immunological cross-reactivity,[1–4] yet they characteristically cause disparate patterns of infection in the human host. For example, *Trichophyton rubrum* (*T. rubrum*) elicits a weak inflammatory response, typically causing chronic or recurrent infection of skin and nails, whilst *T. verrucosum* elicits a brisk inflammatory response associated with rapid resolution and resistance to re-infection. Decreased cell-mediated immunity has been observed in patients with chronic dermatophytoses[5] but does not appear to reflect low levels of circulating antigen, which is detectable in sera from patients with both acute and chronic infection.[6]

Intact cellular immunity is critical for eradication of dermatophytes from the skin.[7,8] Persistence of *T. rubrum* infection in otherwise healthy individuals might therefore result from selective anergy to dermatophyte antigens as previously suggested[9] or reflect the activity of putative dermatophyte-derived lymphocyte-inhibitory factors. In support of these proposed mechanisms, dermatophyte antigen has been shown to suppress cellular immunity in murine models of experimental dermatophyte infection.[10] Moreover, autologous serum from patients with deep or unusually extensive dermatophyte infection has similarly been shown to suppress lymphocyte transformation to recall antigens *in vitro*.[11,12] Because this phenomenon is not observed following successful treatment of infection it suggests the inhibitory effect might be due to circulating dermatophyte-derived blocking factors.

Correspondence: Professor R.J.Hay, Department of Dermatology, Guy's Hospital, St Thomas' Street, London SE1 9RT, U.K.

This study examines cellular immune responses to recall antigens and possible circulating inhibitory factors in patients with chronic *T. rubrum* infection. Lymphocyte transformation to a variety of mitogenic and antigenic stimuli, including a cytoplasmic and exoantigen preparation of *T. rubrum*, was measured in patients with chronic *T. rubrum* infection and compared with controls in the presence of either fetal calf serum or autologus serum. In addition, the ability of *T. rubrum* to suppress lymphoproliferation directly was determined *in vitro*.

Methods

Patients

Fifteen healthy adults (10 males, five females) with culture-proven *T. rubrum* skin infection were recruited. They comprised 13 patients with dry-type tinea pedis and two patients with extensive tinea corporis. All had either failed to respond to, or had relapsed within 3 months of stopping griseofulvin treatment. No patient had been on oral medication, including antifungal treatment, for at least 6 weeks prior to the study. As positive controls 15 age- and sex-matched healthy adults were recruited; all had a previously documented history of inflammatory-type tinea pedis caused by *T. interdigitale*. Despite a high prevalence of atopy in patients predisposed to chronic dermatophyte infection, no patient in our study was atopic.

Fetal cord blood samples ($n = 2$) were used as negative controls.

Isolation of peripheral blood mononuclear leucocytes (PBML)

Blood was collected by venepuncture from each volunteer into sterile universal containers with preservative-free heparin. PBML were isolated from each sample by density-gradient centrifugation using Lymphoprep (Nycomed, U.K.). Lymphocytes stained with eosin were counted using a haemocytometer, and cells resuspended in a concentration of 10^6 cells/ml in RPMI (Gibco, U.K.) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin and penicillin, buffered with 1% Hepes. In addition either 5% heat-inactivated fetal calf serum (FCS) or 5% autologous serum was added prior to use in proliferation assays.

Cultures of Trichophyton rubrum

An isolate of *T. rubrum* was inoculated into autoclaved medium containing sodium β-glycerophosphate, D-glucose and peptone. Cultures were aerated using mechanical stirrers and allowed to grow in 250 ml tissue-culture flasks for 21 days at room temperature. Surface cultures were then harvested by filtration through Whatman's No. 2 filter paper and the supernatants retained. Mycelial suspensions were washed in phosphate-buffered saline (PBS 0·01 M, pH 7·4), homogenized using sterile glass beads (Biospec Products) and the cell extract, cytoplasmic antigen (CA), centrifuged at 1500 *g* for 7 min. Culture supernatant (exoantigen) and CA preparations of *T. rubrum* were dialysed against 100% polyethylene glycol (PEG) and then isometrically dialysed against PBS at 4°C. In addition, dialysates of *Epidermophyton floccosum* and *T. mentagrophytes* were similarly prepared. Protein determinations were estimated by the Coomassie blue method[13] and antigens were stored at −70°C until required. Dialysed uninoculated medium was used as a negative control in each experiment.

Lymphoproliferation assays

Cells (10^5 cells/100 μl) cultured in either 5% FCS or 5% autologous serum, were added to round-bottomed 0·2 ml 96-well microtitre plates (Flow Laboratories, High Wycombe, U.K.) containing either antigen or mitogen (Table 1). Parallel experiments were also performed using EBV-transformed B lymphocytes (M958/ATCC

Table 1. Antigens and mitogens employed in lymphoproliferation assays

Protein	Antigen/mitogen	Concentration (μg/ml)	Source
Purified protein derivative (PPD 289)	Antigen	50–200	Department of Agriculture, Fisheries and Food, Surrey, U.K.
Candida albicans (NCPF 3153)	Antigen	5–500	Mycological Reference Laboratory, Public Health Service Laboratories, Colindale, London, U.K.
TR cytoplasmic antigen	Antigen	0·046–460	As described
TR exoantigen	Antigen	0·025–250	As described
Phytohaemagglutinin (PHA, L8754)	Mitogen	20–200	Sigma, U.K

Laboratories, U.S.A.) in place of PBML. Cultures were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for 4 days. On day 4, 0·2 μCi ^{3}H-thymidine (Amersham, specific activity 185 GBq/mmol) was added to each well and cells were incubated for a further 16 h before harvesting. Cells were harvested on to glass-fibre filter paper (G-10, Inotech, New Hampshire) using a semi-automatic 96-well cell harvester (Inotech), dried at 40°C and placed in vials containing scintillation fluid (Wallac, Sweden). Radioactive uptake by cells was measured using a liquid scintillation beta counter (Wallac). All experiments were performed in triplicate and a mean value expressed as disintegrations per minute – background (d.p.m. – Δ). Statistical analysis was performed on all results using an unpaired Student's *t*-test.

HLA-DR expression by PBML using a double-labelling immunofluorescence technique

PBML were incubated for 3 days with either RPMI, PHA, PHA and TR exoantigen, or TR exoantigen alone, and then fixed in 0·01% azide in PBS. Non-specific binding was blocked using heat-inactivated normal human serum, and cells were labelled first with unconjugated monoclonal antibody (MAb) to HLA-DR (L243)(ATCC, U.S.A.) and then with fluorescein isothiocyanate-conjugated (FITC) polyclonal goat anti-mouse immunoglobulin antibody (Becton Dickinson, Mount View, CA, U.S.A.). Following incubation with normal mouse serum, cells were subsequently labelled with a phycoerythrin-conjugated MAb to CD4 or CD8 (Becton Dickinson). Samples were washed twice between each step. Controls included irrelevant antibodies for both red and green fluorescence, and single-stain analysis for each antibody was used. Cytofluorometric analysis, counting 5×10^3 cells per sample, was performed on a fluorescence-activated cell sorter (FACStar, Becton Dickinson). Appropriate forward and 90 light scatter gates were set to exclude dead cells and non-lymphoid cells. Positive cells were determined by setting a threshold with reference to the relevant negative controls.

Results

Using this protocol, optimal (highest mean value) lymphoproliferative responses were obtained at the following concentrations: phytohaemagglutinin 40 μg/ml, purified protein derivative 100 μg/ml, *T. rubrum* (TR) exoantigen 2·5 μg/ml, TR CA 10 μg/ml and *Candida albicans* 56 μg/ml. Individual responses to antigens and mitogens varied, but no one individual was a consistently low responder to all stimuli. No significant difference in mean lymphoproliferative response to any mitogen or antigen was found between patients and controls when compared using a Student's unpaired *t*-test (Fig. 1). Furthermore, equivalent lymphoproliferative responses to all antigens were seen using either FCS or autologous serum from patients with chronic *T. rubrum* infection.

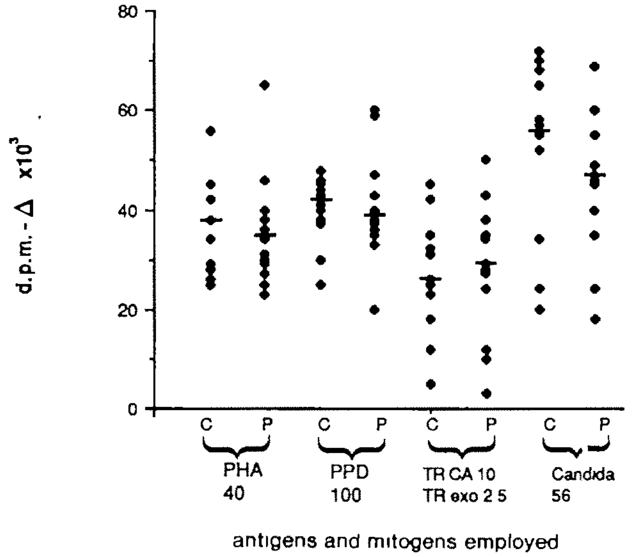

Figure 1. Scattergraph demonstrating lymphocyte proliferation to recall antigens and PHA. a comparison of patients (P) with chronic *T. rubrum* infection and controls (C). Statistical analysis performed using the Student's unpaired *t*-test demonstrates no significant difference between the groups.

These results indicate that PBML from patients with chronic *T. rubrum* infection respond normally to PHA and to recall antigens, as well as to both an exoantigen and a cytoplasmic antigen preparation of TR. The possibility that TR preparations had a mitogenic effect was excluded by the absence of lymphoproliferation using PBML from fetal cord blood (Fig. 2), although these data do not exclude a possible superantigen effect of TR preparations. There was no evidence to support the presence of a circulating inhibitory factor in sera from patients with chronic superficial *T. rubrum* infection.

Concentration-dependent inhibition of lymphoproliferative responses by TR (Fig. 3)

PBML from five patients previously found to have strong proliferative responses to TR preparations were used, and each experiment was performed in triplicate. Maximal stimulation of PBML by TR CA was observed at

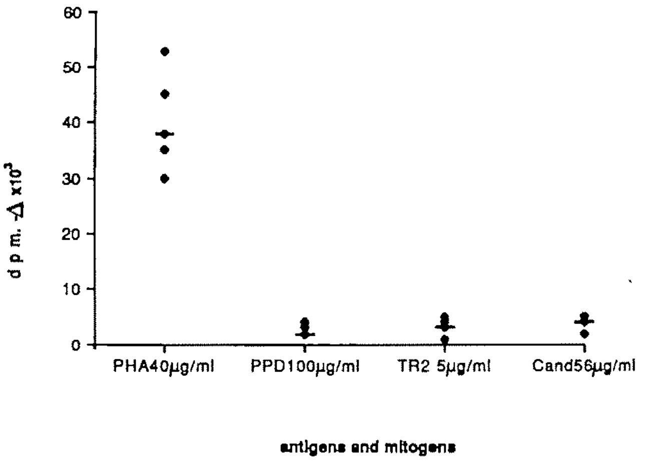

Figure 2. Scatter graph demonstrating proliferation of PBML from fetal cord blood to PHA. Proliferation was not observed with any antigen, including TR exoantigen and TR cytoplasmic antigen.

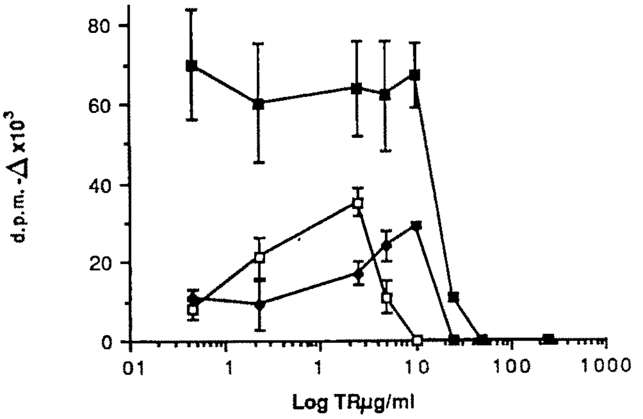

Figure 3. Line graph demonstrating lymphoproliferation to TR exoantigen (□) and TR cytoplasmic antigen (◆) Maximal proliferation is observed at 2·5 µg/ml and 10 µg/ml, respectively. Conversely, complete inhibition of lymphoproliferation is seen at >10 µg/ml TR exoantigen and >25 µg/ml TR cytoplasmic antigen. Inhibition of EBV-transformed B lymphocytes by equivalent concentrations of TR inhibitor is similarly observed (■).

protein concentrations of 10 µg/ml and by TR exoantigen at 2·5 µg/ml. In contrast, at concentrations >25 µg/ml and >10 µg/ml, respectively, complete inhibition of proliferation was observed using both PBML and EBV-transformed B lymphocytes. No inhibition was observed using dialysed uninoculated culture medium alone. This suggests a dermatophyte inhibitory factor (DIF) is present in both preparations. TR exoantigen preparation has both increased stimulatory and inhibitory effects compared with equivalent protein concentrations of TR CA, suggesting that both major stimulatory antigens and immunoinhibitory factors derived from *T. rubrum* are secreted by the organism.

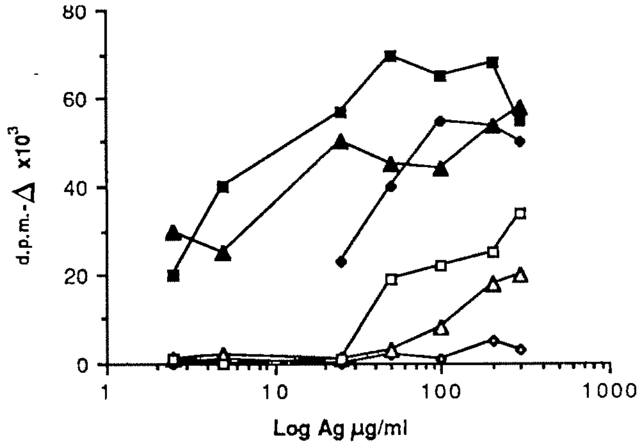

Figure 4. Demonstration of dose-related lymphoproliferation to PHA(□), PPD(△) and *C. albicans*(◇) with (open figures) and without (solid figures) the presence of TR inhibitor. Inhibition of lymphoproliferation to each antigen and PHA is observed on addition of TR inhibitor. Escape from inhibition occurs when sufficiently high concentrations of PHA and PPD are employed (PHA>100 µg/ml, PPD>200 µg/ml, *C albicans*>168 µg/ml)

Mechanism of inhibition

Lymphoproliferation to recall antigens and PHA inhibited by TR (Fig. 4). Experiments were performed in triplicate on PBML from five patients. Very similar patterns of response were observed in each case, but statistical analysis of pooled data was not performed due to the large inter-patient variability in lymphoproliferative responses to antigen preparations. Figure 4 represents the mean of three experiments performed on PBML from one patient.

Lymphoproliferation to recall antigens and to PHA was abrogated by co-culturing with DIF (TR CA >25 µg/ml and TR exoantigen >10 µg/ml). This inhibitory effect was not observed using dialysed uninoculated medium. Increasing concentrations of mitogen or antigen titrated against a fixed concentration of DIF overrode the inhibitory effect, indicating that it was not due to loss of cell viability. This is supported by the demonstration that the inhibitory effect of DIF on lymphocytes could be rapidly reversed by washing inhibited lymphocytes in PBS and resuspending them in RPMI containing either antigen or mitogen (data not shown).

HLA-DR expression by lymphocytes cultured with DIF and PHA (Table 2). Three experiments were performed on PBML from one patient. Results represent the mean of these data. HLA-DR expression (measured as mean intensity of fluorescence using FITC-DR) was markedly upregulated on lymphocytes stimulated with PHA and

Table 2. Flow cytometric analysis of HLA-DR antigen expression on CD4$^+$ PBML

Culture medium	RPMI	PHA	PHA and TR inhibitor	TR inhibitor
FITC-DR mean intensity of fluorescence	53·5	547	701	83·3
d.p.m. (mean)	500	56,000	8000	243

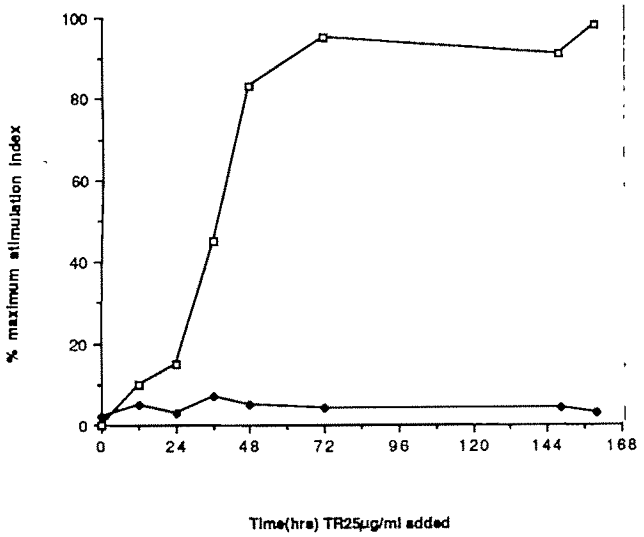

Figure 5. Addition of TR inhibitor to cultured lymphocytes(□) at t= 0–160 h. Maximal inhibition of antigen-driven lymphoproliferation is seen when TR inhibitor is added at $t<24$ h. EBV-transformed B lymphocytes(◆) are consistently inhibited by addition of TR (t= 0–160 h).

on lymphocytes co-cultured with both PHA and DIF, despite inhibition of cellular proliferation. In contrast, lymphocytes cultured in RPMI or DIF alone did not express HLA-DR, suggesting that some events required for T-lymphocyte activation, such as DR synthesis, proceed normally in the presence of DIF and PHA.

Time-dependent effect of TR inhibition on lymphoproliferation (Fig. 5). Lymphocytes cultured with PPD were maximally inhibited when DIF was added to the culture at t=0–24 h. Thereafter, addition of DIF had no significant effect on lymphoproliferation. In contrast, addition of DIF to EBV-transformed B lymphocytes consistently inhibited lymphoproliferation at all times (t=0–160 h).

Discussion

Lymphocyte transformation *in vitro* has previously been employed to examine cellular immunity in human dermatophytoses. Although some studies suggest a selective impairment of lymphoproliferation to dermatophyte antigens in patients with chronic dermatophyte infection,[9] most studies have shown normal cellular responses to all recall antigens, including dermatophyte antigen.[14] Our data, which demonstrate normal lymphocyte transformation to TR antigen in patients with chronic dermatophytosis, are in agreement with recent studies,[14] and do not therefore support the hypothesis that individuals predisposed to chronic dermatophyte infection exhibit selective anergy to *T. rubrum* antigen. This does not exclude, however, the possibility that lymphocyte function may be defective in these individuals.

Our study demonstrates that a dermatophyte-derived inhibitory factor, or factors (DIF), present in both TR CA and supernatant (exoantigen) preparations inhibits lymphocyte proliferation to a variety of recall antigens *in vitro*. Studies of murine dermatophytoses similarly suggest that dermatophyte antigen may directly suppress lymphocyte function.[10] Because dermatophyte antigen can be demonstrated both in the serum[6] and in the epidermis during human cutaneous infection,[15] it is possible that a local inhibitory effect on epidermal lymphocytes might explain the inability of patients chronically infected with *T. rubrum* to eradicate fungus from the skin, even in the presence of normal cell-mediated immunity. The observation that patients with normal *in vitro* lymphocyte transformation to *T. rubrum* characteristically lack delayed-type hypersensitivity responses to intradermal dermatophyte antigen is also consistent with this hypothesis. It is not currently known, however, whether dermatophyte antigen is present in the epidermis in sufficient quantities to inhibit lymphoproliferation *in vivo*. Nor is it known whether a putative local immunosuppressive effect is of any clinical relevance in dermatophyte infection.

Inhibition of lymphoproliferation was observed in this study by addition of TR preparation to EBV-transformed B lymphocytes and also to lymphocytes stimulated with PHA. These data suggest that the target cell for the inhibitory effect *in vitro* may be the lymphocyte rather than the antigen-presenting cell. The observation that lymphocytes co-cultured with DIF and PHA express HLA-DR antigen, a surface marker of T-cell activation, also suggests the inhibitory effect may occur at a step common to many dividing cells, independent of antigen presentation. Because the inhibitory effect is readily reversible by washing, a cell-surface interaction may be more likely than an intracellular event. We cannot currently explain why EBV-transformed B lymphocytes are consistently inhibited by DIF whilst antigen-driven lymphoproliferation escapes the inhibitory effect after a critical 24 h in culture, but consider that this observation may provide an important clue to the mechanism of inhibition.

We were unable to demonstrate an inhibitory effect on lymphocyte proliferation using autologous serum from any patient with chronic superficial *T. rubrum* infection. Serum inhibitory factors previously described,[11,12] might therefore reflect particularly high levels of circulating antigen in patients with unusually extensive disease. Reduced proliferation of spleen and lymph node lymphocytes observed in mice with *T. quinckeanum* infection in the presence of autologous serum correlated with the level of circulating dermatophyte antigen,[10] suggesting that antigen load may be critical for an inhibitory effect to be observed *in vivo*.

We have observed similar lymphocyte inhibition using exoantigen preparations of *T. mentagrophytes* and *E. floccosum* (unpublished data), two organisms typically associated with more inflammatory infections. Moreover, inhibitory effects on lymphoproliferation strikingly similar to our own[16] were reported using mannan extracted from both yeasts[17] and *T. rubrum*,[18] suggesting that many fungi may synthesize factors with lymphocyte-inhibitory properties. The outcome of an infective episode may thus be determined by the net effect of both dermatophyte-derived inhibitory and stimulatory factors.

T. rubrum accounts for approximately 90% of chronic superficial dermatophyte infections in the human host. Although some patients appear to have a particular susceptibility to chronic and recurrent disease, our *in vitro* data suggests this is unlikely to result from an endogenous inability to respond to dermatophyte antigen. Instead, it is possible that persistence of *T. rubrum* in the human host could result from a local inhibitory effect of dermatophyte antigen *in vivo*, as demonstrated to occur *in vitro*, on lymphocytes recruited to the epidermis. This putative suppression of lymphocyte activity at the site of infection might thus prevent eradication of fungus from the skin, even in the presence of normal *in vitro* cellular responses to dermatophyte antigen. Further research is necessary to establish whether the inhibitory properties of dermatophyte antigen observed *in vitro* do have a role to play in the chronicity of human cutaneous fungal infections.

Acknowledgment

This work was sponsored by a research grant donated by Sandoz Pharmaceuticals, U.K.

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British Journal of Dermatology (1992) 127, 239–246.

Characterization of inflammatory infiltrates in male pattern alopecia: implications for pathogenesis

CHRISTINE JAWORSKY, A.M.KLIGMAN AND G.F.MURPHY
Duhring Research Laboratories, Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.

Accepted for publication 3 January 1992

Summary

Hair-bearing, transitional, and alopecic scalp from three males and one female with progressive pattern alopecia were examined. Ultrastructural studies disclosed measurable thickening of the follicular adventitial sheaths of transitional and alopecic zones compared with those in the non-alopecic zones. This finding was associated with mast cell degranulation and fibroblast activation within the fibrous sheaths. Immunohistochemically, control biopsies were devoid of follicular inflammation ($n = 3$), while transitional regions consistently showed the presence of activated T-cell infiltrates about the lower portions of follicular infundibula. These infiltrates were associated with the induction of class II antigens on the endothelial linings of venules within follicular adventitia and with apparent hyperplasia of follicular dendritic cells displaying the CD1 epitope. Inflammatory cells infiltrated the region of the follicular bulge, the putative source of stem cells in cycling follicles. The data suggest that progressive fibrosis of the perifollicular sheath occurs in lesions of pattern alopecia, and may begin with T-cell infiltration of follicular stem cell epithelium. Injury to follicular stem cell epithelium and/or thickening of adventitial sheaths may impair normal pilar cycling and result in hair loss.

Pattern alopecia (PA)[1–3] is the hormonally driven hair loss in genetically predisposed adults. Initially it affects the frontal regions and the vertex of the scalp, and may progress to involve a large area of the scalp, but typically preserves the occipital fringe.[4] Participation of androgenic steroids in the genesis and perpetuation of PA has been addressed by studies focused at levels of circulating post-pubertal androgens,[5] androgen conversion to active metabolites at target sites,[6] and androgen-binding serum proteins.[7,8] The possibility that immune mechanisms may contribute to PA is based upon anecdotal symptoms of pruritus in evolving disease, preliminary evidence of the efficacy of immunomodulatory therapy,[9] and naturally occurring follicular inflammatory disorders that result in localized or diffuse alopecia.[10–13] Because endogenous steroid hormones have been shown to influence cytokine and cellular immune responses,[14,15] it is plausible that both hormonal and immunoregulatory mechanisms act synergistically to potentiate follicular injury in PA. The majority of the morphological studies that have investigated this issue, however, have directed attention to analysis of alopecic skin, where end-stage injury fails to reveal early alterations fundamental to pathogenesis.

There is increasing awareness of the presence of immunocompetent cells in the external root sheath of the normal follicle. The infundibulum of the cycling hair follicle is richly endowed with CD1 and HLA-DR-positive dendritic cells.[16] These cells have not as yet been quantified in follicular epithelium as they have in the interfollicular epithelium using ATPase-stained epidermal sheets. Difficulties in enumerating follicular dendritic cells relate at least in part to the tubular three-dimensional architecture of this site. None the less, significant numbers of such cells can be seen in the follicular epithelium by using immunohistochemical stains. The adventitial sheath that envelops the follicle contains an elaborate plexus of arterioles and venules that is contiguous with the microvasculature of the papillary dermis.[17] Post-capillary venules within this sheath are surrounded by resident connective tissue mast cells, monocyte macrophages, and occasional T lymphocytes. Thus, the hair follicle is equipped with a full complement of cells to promote and perpetuate both afferent and efferent immune responses. Although existing data are incomplete with respect to cytokine produc-

Correspondence: Dr C.Jaworsky, Clinical Research Building, 422 Curie Boulevard, Philadelphia, PA 19104-6065, U.S.A.

tion by follicular keratinocytes, it is probable that they will prove to subserve immunoregulatory functions akin to their epidermal counterparts. Indeed, recent data indicate that the infundibulum is an early target of acute and transient T-cell responses mediated by both CD4 and CD8-positive effector cells.[18,19]

In the present study, we have examined biopsies from three scalp areas in four patients with progressive PA: alopecic, hair-bearing skin, and the thinning transitional areas. We compared these findings with occipital scalp from three control subjects. Our approach was influenced by recent evidence indicating that follicular stem cells reside in the 'bulge' region of the follicle, a zone directly beneath the orifice of the sebaceous duct.[20] We found that evolving PA is associated with persistent infiltrates of activated T cells which predominate in the transitional scalp areas. Moreover, follicular T-cell infiltration selectively involved the putative stem cell region of affected follicles. Such changes were associated with degranulation of follicular adventitial mast cells and with evidence of enhanced collagen production by nearby fibroblasts. These observations imply immune mechanisms in the natural evolution of PA.

Methods

Patients

Four healthy adult subjects (three males and one female) underwent elective surgical reduction of vertex scalp skin for progressive PA. Their ages ranged from 25 to 67 years. There were no clinical signs of primary scalp inflammation or associated dermatitis/folliculitis. Biopsies were obtained from (i) alopecic zones, characterized by complete hair loss; (ii) peripheral regions (within 1–2 mm of surgical margins, where hair density was greatest; and (iii) transitional regions of diminished hair density separating (i) and (ii). A total of 18 specimens were evaluated.

Morphological analysis

Sections for light microscopy (1 μm) were prepared by fixing minced tissue in 4% glutaraldehyde for 4–6 h at 4°C, rinsing in 0.1 M sodium cacadylate buffer (pH 7·4), post-fixing in 2% osmium tetroxide, serial dehydration in graded ethanol, and embedding in Epon. Original samples were subdivided with the assistance of stereoscopic microscopy to maximize preservation of intact follicles. Regions of interest were then selected for ultrathin sections prepared with an LKB ultramicrotome. After staining with uranyl acetate and lead citrate, specimens were examined with a Hitachi HU12A transmission-electron microscope. Control biopsies were obtained from clinically normal, hair-bearing occipital scalp of three healthy young adult males without alopecia, and without a family history of PA.

Immunohistochemical analysis

Samples selected for immunohistochemistry were stored briefly in Michel's solution (24–48 h), rinsed in buffer, and snap-frozen in Tissue-Tek embedding medium (Miles Laboratories, Napperville, IL, U.S.A.). Cryostat sections 4 μm thick were then reacted with primary monoclonal antibodies for 1 h at 37°C. Epitopes were identified and their respective antisera consisted of CD5 (mature peripheral T cells)–Leu 1; CD8 (cytotoxic/suppressor T cells)–Leu 2a; CD4 (helper/inducer T cells; some activated macrophages/Langerhans cells)–Leu 3a; CD1 (Langerhans cells)–Leu 6; HLA-DR (Langerhans cells, activated and interferon-γ-stimulated mononuclear, endothelial, and dendritic cells; B cells)–anti-HLA-DR (all Becton Dickinson, Mountain View, CA); and TAC (interleukin 2 receptor-bearing cells)–anti-TAC (Dakopatts, Santa Barbara, CA). Staining was achieved by a three-step avidin–biotin peroxidase procedure (Vector Labs, Inc., Burlingame, CA, U.S.A.) with 3,3′-diaminobenzidine (DAB) as the chromogenic substrate.[21–24] Isotype matched controls were performed to exclude non-specific binding of primary antisera. In cases where expression of different epitopes required numerical or structural comparison, adjacent sections were stained with the relevant primary antibodies.

Results

Morphological and ultrastructural changes

Changes noted by 1-μm section analysis related to growth patterns of hair follicles and to changes in the supporting fibrovascular sheaths enveloping each hair follicle. Biopsies from hair-bearing scalp of normal non-alopecic young adults (controls) contained in excess of 75% anagen follicles in sections, similar to findings from non-alopecic scalp obtained from the hair-bearing zones of subjects experiencing progressive PA. By contrast, the biopsies from transitional scalps showed a significant reduction in the percentage of hair in active growth phases (anagen) to approximately 50%. In alopecic scalp biopsies, anagen hair follicles were rare, and for the most part were replaced by empty fibrous tracts, the residua of previous cycling elements.

Corresponding changes were also noted in follicular adventitial sheaths, the delicate supporting fibrovascular networks surrounding hair follicles. In control specimens and non-alopecic scalps of patients with PA the fibrovascular sheaths were delicate and contained fully granulated mast cells by light and electron microscopy. Inflammatory cells were rarely encountered within the sheaths or adjacent epithelium. Sheath

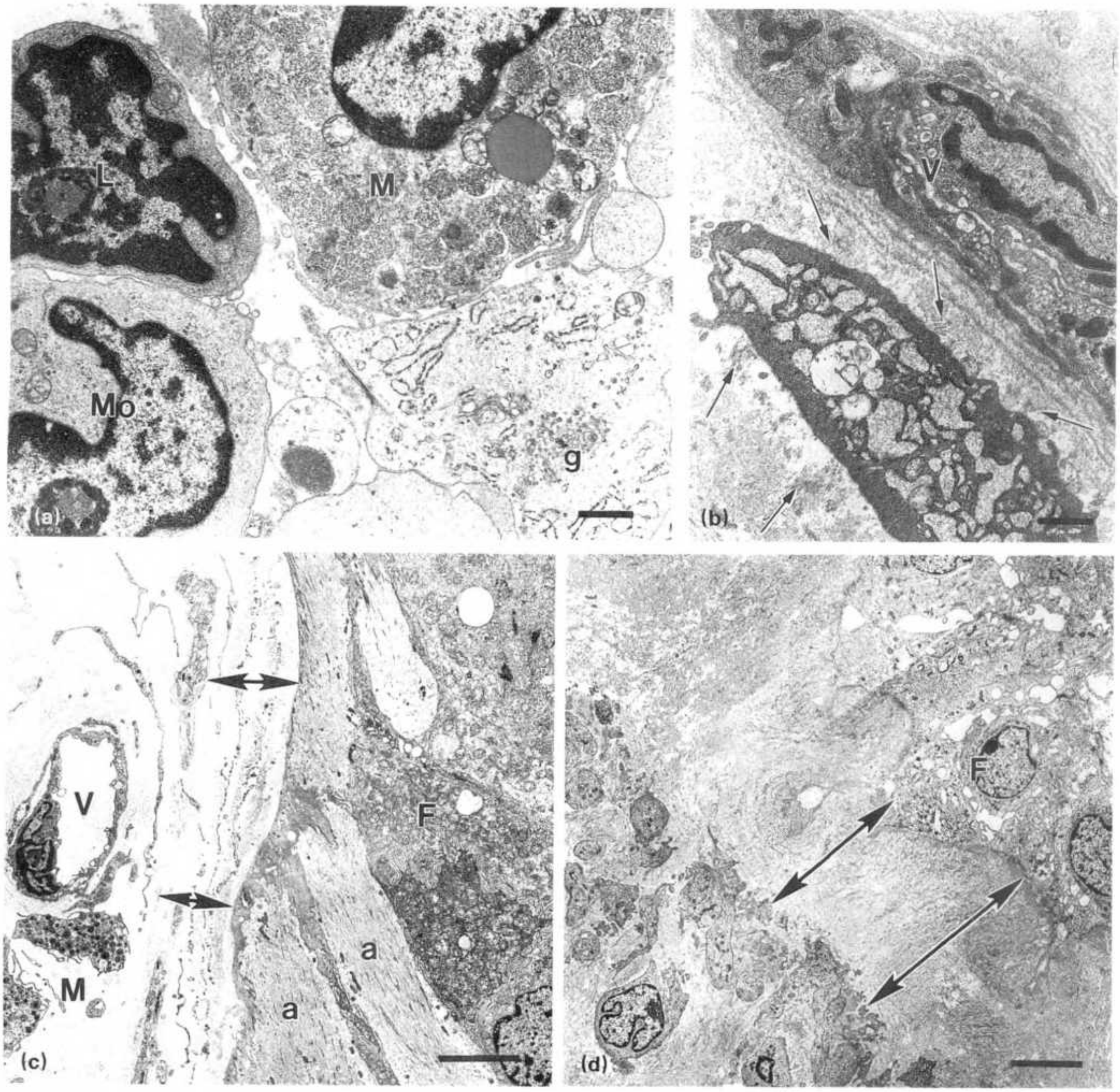

Figure 1. Transmission-electron microscopy of lower follicular infundibulum in evolving PA. Inflammatory infiltrates in transitional skin (a) were composed predominantly of lymphocytes and monocytoid cells (Mo) (representing activated T cells or true monocytes) closely associated with actively degranulating mast cells (M) and fibroblasts with prominent golgi zones (g) and rough endoplasmic reticula dilated by granular material. These fibroblasts (b) also displayed evidence of peripheral secretion of procollagen-type filaments (arrows) and were present in increased numbers within widened adventitial sheaths also containing venules (V) lined by enlarged endothelial cells. Whereas normal control and non-alopecic scalp (c) showed relatively thin follicular sheaths (double arrows) in the vicinity of the follicular stem cell (F) region (near the sebaceous orifice and arrector pili muscle [a] insertion site), alopecic scalp (d) was characterized by sheaths widened by densely packed collagen bundles (double arrows). Note fully granulated mast cell (M) in panel (c). (a. × 10,000; b. × 5000; c and d. × 2200. Bar = 1 μm (a), 2 μm (b), 5 μm (c, d)).

fibroblasts had collapsed and inconspicuous cisternae of rough endoplasmic reticulum, were indicative of a quiescent state. The diameter of the sheaths ranged between 18 and 28 μm, with an average of 22 μm (Fig. 1). The sheaths were measured with an ocular micrometer from the base of follicular epithelial cells to the outermost layers of the adventitial sheaths in 54 fields.

In transitional scalp, the thin zone of partial hair loss separating non-alopecic and alopecic scalp, findings differed dramatically from control and non-alopecic biopsies. Although ultrastructural analysis failed to demonstrate cytotoxic alterations of the follicular epithelium, it consistently disclosed close associations between infiltrating mononuclear cells and actively degranulating mast cells within follicular sheaths (Fig. 1a). Moreover, mast cells were often associated with fibroblasts exhibiting hyperplasia of biosynthetic organelles and cisternae of rough endoplasmic reticulum widened by granular amorphous material (Fig. 1a and b). These fibroblasts also displayed plasma membrane-associated fibrils consistent with procollagen secretion[25] and were regularly observed within sheaths widened by apparently increased deposits of mature collagen bundles. The follicular sheath diameter in these zones ranges from 15

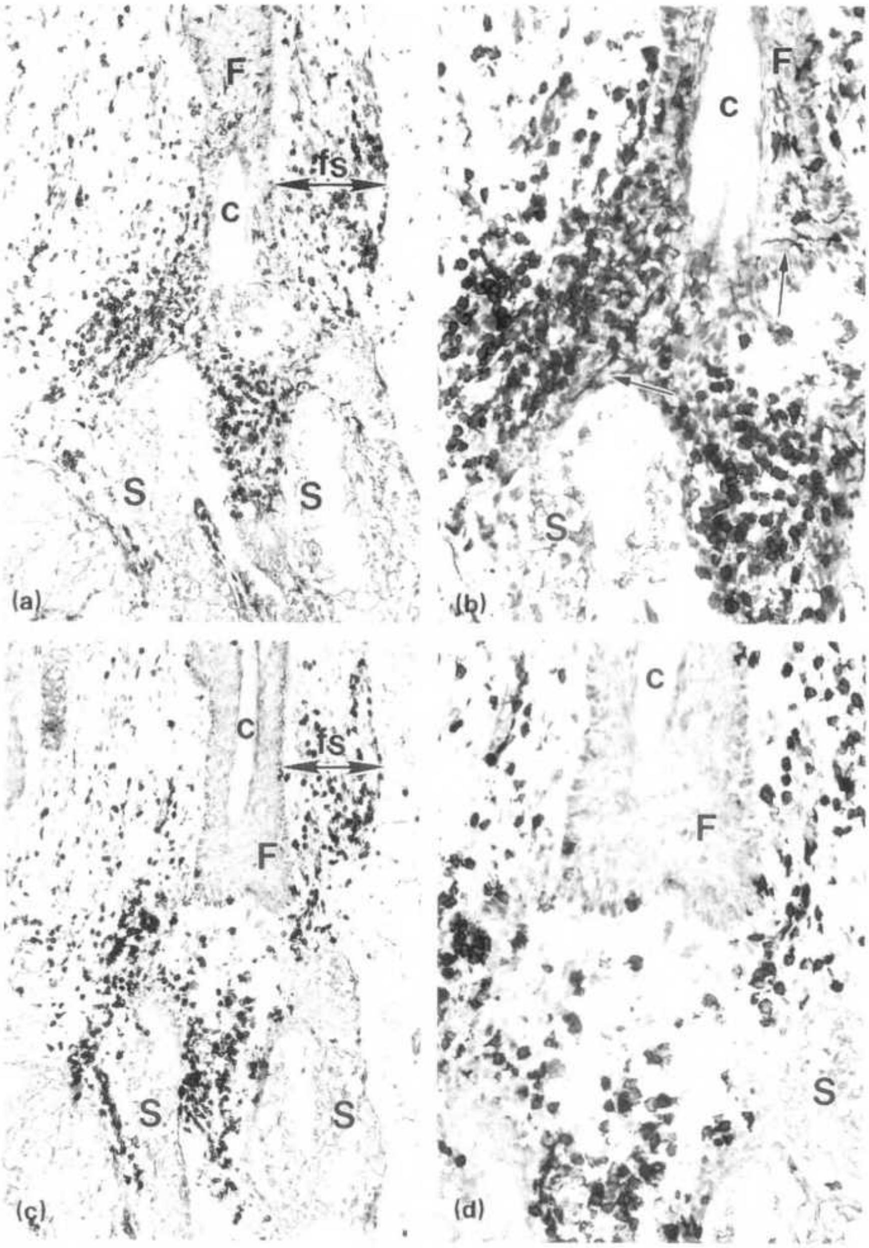

Figure 2. Immunohistochemistry of T-cell follicular infiltration in PA: transitional scalp. (a) and (b) depict low and high magnifications of CD4-positive T cells, and (c) and (d) represent similar views of adjacent section reacted for CD8- positive T cells. Note localization of infiltrates about the lower follicular infundibulum (F) near the origins of sebaceous lobules (S). In addition to follicular epithelial infiltration (b and d), adjacent follicular adventitial sheaths (fs, double arrows) are markedly expanded by T cells (a and c). Intrafollicular dendritic cells show focal induction of CD4 epitope (b, arrows). (c = pilar canal.) (a and c, × 100; b and d, × 200).

to 61·6 μm, with an average of 39 μm (Fig. 1c and d) in 32 fields.

The majority of follicles of alopecic scalp demonstrated replacement of underlying trichogenic elements by fibrotic sheath residua ('fibrous tracts') containing degranulating mast cells and ectatic blood vessels. Incidental light microscopic findings included widened follicular infundibula plugged by laminated keratin and variable sized sebaceous lobules. Occasional sheath fibroblasts showed continued evidence of heightened matrix synthesis, although these findings were not as pronounced or widespread as those observed in transitional scalp.

Immunohistochemical findings

Control specimens showed rare Leu 1-positive T cells in the superficial dermis without preferential follicular distribution. These cells were too sparse to quantify with regard to expression of CD4 and CD8 epitopes, but both subsets were represented. CD1-positive dendritic cells were evenly distributed in interfollicular epidermis and within the external root sheath of follicular infundibula. Approximately 75% of these cells expressed HLA-DR and none displayed CD4. Endothelium lining occasional dermal and perifollicular vessels, rare dermal dendritic cells, and follicular adventitial dendritic cells demonstrated slight and variable reactivity for HLA-DR. Interleukin 2 receptor expression was not detected. Immunohistochemical analysis of non-alopecic scalp was similar to that of control specimens, with the exception of occasional anagen follicles showing small numbers of Leu 1-positive cells around and within the epithelium and follicular sheath of the lower infundibulum. The CD4:CD8 ratio of these T cells approximated 1:1 and occasional cells expressed HLA-DR.

In biopsies of transitional scalp, lower portions of follicular infundibula showed extensive infiltration by mononuclear cells, the majority of which (>95%) were Leu 1-positive T cells (Fig. 2). The infiltrates were centred around infundibular epithelium in the vicinity of the sebaceous duct orifice, a region recently identified as the site of follicular stem cells in murine follicles.[20] The lowermost bulbar region of the follicle was uninvolved. The CD4:CD8 ratio of these infiltrates was 2–3:1, and the majority (>90%) displayed some degree of membrane HLA-DR and interleukin 2 receptor expression.

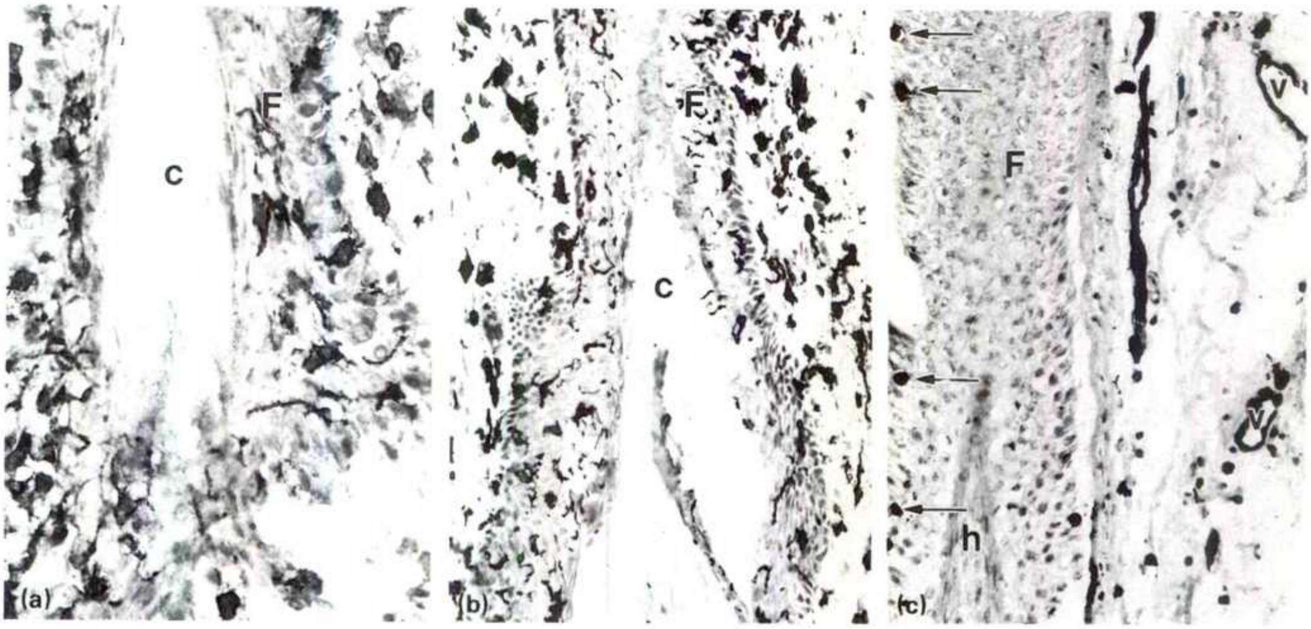

Figure 3. Immunohistochemistry of follicular dendritic and endothelial cells in PA; transitional scalp. Marked hyperplasia of CD1-positive dendritic cells (a) was observed within follicles (F) and adjacent adventitial sheaths at sites of T-cell infiltration (lower infundibulum). The basaloid follicular protuberance to the right of the pilar canal (c) resembles the stem cell bulge recently described in murine follicles. Marked induction of HLA-DR was also observed in follicular and perifollicular dendritic and mononuclear cells (b). Subjacent, non-infiltrated portions of affected follicles (c) demonstrated prominent HLA-DR expression by swollen endothelial cells lining all vessels (V) within the adventitial sheath, and occasional HLA-DR-positive mononuclear cells within outer follicular epithelium (arrows). These changes correlated with mast cell degranulation diffusely throughout the adventitial sheath (h = hair shaft) (a, × 400; b and c, × 200).

CD1 and HLA-DR-positive dendritic cells appeared to be increased within follicular epithelium. The finding of increased numbers of follicular Langerhans cells, although subjective, is one noted in studies of other alopecias.[26,27] The adjacent adventitial sheaths were involved by T-cell infiltration (Fig. 3a and b). In contrast with control and non-alopecic scalp, occasional dendritic cells were also detected within the epithelium of the underlying isthmus and bulb. These dendritic cells also expressed CD4, a finding not detected in control tissue, and associated with immunologically stimulated dendritic cell populations.[28] In addition, CD1-negative, HLA-DR-positive bipolar and dendritic cells (possibly representing histiocytes or fibroblasts) were increased in number within widened follicular sheaths. Endothelial cells lining the perifollicular microvasculature were strongly and uniformly positive for HLA-DR (Fig. 3c).

In alopecic scalp dendritic cell hyperplasia was not observed and HLA-DR expression by dendritic cells and endothelial cells resembled that of control and non-alopecic scalp. Inflammatory infiltrates were scarce.

Discussion

The findings disclosed by detailed analysis of scalp skin variably involved with actively progressive pattern alopecia suggest that (i) the pathogenesis of PA involves T cell and dendritic cell responses at the lowermost level of the follicular infundibulum; (ii) mast cell degranulation within adventitial sheaths is a prominent feature of this phenomenon; and (iii) fibroblast activation and resultant sheath fibrosis may contribute to impaired pilar cycling and permanent hair loss.

Previous investigations of the pathogenesis of PA have concentrated on alopecic skin, a terminal stage in this disorder, and were unlikely to reveal early alterations leading to end-stage injury. Our findings suggest that disturbances of the follicle-associated immune system, a complement of immunoregulatory cells and soluble factors analogous to the skin-associated immune system of interfollicular epidermis, may represent critical early events in the genesis of PA. Although it might be reasoned that inflammatory events capable of inducing permanent alopecia should be centred about matrix cells of the hair bulb, and not the lower infundibulum, the immunoarchitecture of many alopecias does not support this notion. For example, whereas permanent alopecia associated with lichen planopilaris and lupus erythematosus characteristically involves inflammation around the infundibular epithelium, the frequently reversible hair loss of alopecia areata typically results from lymphocytic infiltration of the bulbar matrix.[29] A possible explanation for these observations is derived from recent experimental evidence that follicular stem cells reside as a specialized subpopulation of cells sequestered directly beneath the orifice of the sebaceous duct in the vicinity of the arrector pili muscle insertion site.[20] This region is the precise site of T-cell localization in follicles in disorders resulting in permanent hair loss, and in transitional zones of PA. Although we did not observe evidence of direct cytotoxicity to stem-cell populations, it is possible that soluble mediators elaborated during the inflammatory cascade may produce a local environment deleterious to cyclic stimulation of stem cells.[30] Further evidence for a role of inflammation in PA derives from the clinical and experimental responses to drugs such as cyclosporin A[31] and minoxidil[32] which potentially exert local anti-inflammatory effects as part of their diverse spectra of therapeutic action.[33,34] Interestingly, minoxidil has also been demonstrated to inhibit hydroxylysine production as well as exerting antiproliferative effects on skin fibroblasts,[35] both of potential relevance to alterations in the follicular sheath that appeared to accompany T-cell infiltration in PA.

Mast cell degranulation was a prominent feature in sheaths of affected follicles, and was associated with ultrastructural evidence of biosynthetic activation of fibroblasts resulting in sheath widening and fibrosis. Mast cell granules have recently been demonstrated to contain cytokines, such as tumour necrosis factor-α capable of inducing pro-inflammatory cellular activation at the level of microvascular endothelium.[36] In PA, persistent T-cell infiltration and mast cell degranulation are also associated with evidence of microvascular activation (HLA-DR expression). In addition to initiation and perpetuation of inflammatory reactions,[37,38] mast cell products have also been implicated in the induction of cutaneous fibroplasia.[39-41] In PA, fibrosis of follicular sheaths induced by similar mechanisms may result in a non-compliant structural relationship between matrix elements and forming trichogenic epithelium during early anagen phase. With chronicity, such factors could contribute to permanent hair loss, leaving behind non-trichogenic superficial portions of infundibular epithelium associated with scarred underlying tracts formerly occupied by intact follicles.

Figure 4 summarizes a hypothetical sequence, extrapolated from the present data, which attempts to unify the roles of inflammation and fibroplasia in the evolution of PA. Integral to this hypothesis is recognition of two elements critical to normal pilar cycling: (i) integrity and responsiveness of lower infundibular stem cells from

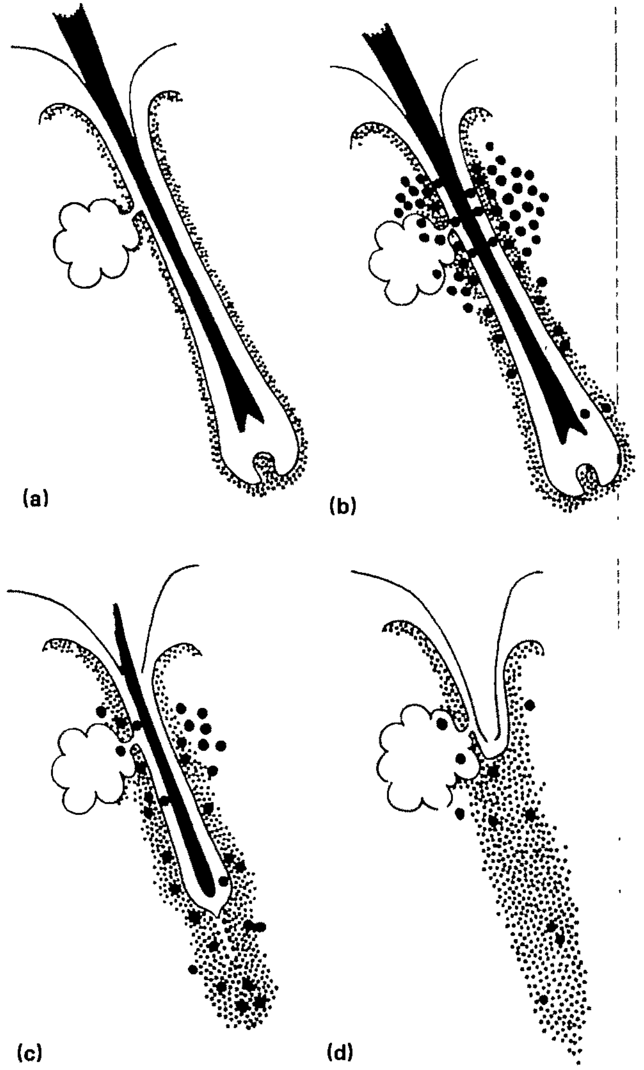

Figure 4. Schematic diagram of evolution of PA Normal anagen follicles (a) become infiltrated by activated T cells which are preferentially distributed within the epithelium and the adventitial sheath at the level of the lower infundibulum, near putative stem cell populations (b). Persistence of T cells within the sheath (c) is associated with mast cell degranulation and induction of collagen synthesis by sheath fibroblasts, resulting in widening and fibrosis with chronicity. The end-stage follicle (d) is characterized by a fibrous tract representing sclerotic sheath remnants, and ectatic superficial infundibular epithelium incapable of trichogenesis

which new anagen follicles are generated, and (ii) patency and compliance of the fibrous sheath into which forming hairs descend. Our observations suggest the possibility that T-cell inflammation and associated mast-cell degranulation centred around the stem-cell regions of follicles collaborate with sheath fibroplasia to permanently impair proliferative and structural components fundamental to normal trichogenesis. These findings raise questions concerning initiating events and inductive factors of follicular inflammation, and the role of immunomodulation in the treatment and prevention of PA. Further studies are necessary to answer these questions and will require examination of the early and subclinical evolutionary stages to delineate the pathogenesis of this common condition.

Acknowledgments

Dr Waine C.Johnson graciously provided the clinical specimens that made this study possible. This study was supported by grants CA 40358 and AR 39674 from the National Institutes of Health.

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British Journal of Dermatology (1992) 127, 247–253.

Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage

D.DARR, S.COMBS, S.DUNSTON,* T.MANNING* AND S.PINNELL

Duke University Medical Center, Division of Dermatology, Durham, NC, U.S.A.

**NCSU School of Veterinary Medicine, Raleigh, NC, U.S.A.*

Accepted for publication 8 April 1992

Summary Ultraviolet radiation damage to the skin is due, in part, to the generation of reactive oxygen species. Vitamin C (L-ascorbic acid) functions as a biological co-factor and antioxidant due to its reducing properties. Topical application of vitamin C has been shown to elevate significantly cutaneous levels of this vitamin in pigs, and this correlates with protection of the skin from UVB damage as measured by erythema and sunburn cell formation. This protection is biological and due to the reducing properties of the molecule. Further, we provide evidence that the vitamin C levels of the skin can be severely depleted after UV irradiation, which would lower this organ's innate protective mechanism as well as leaving it at risk of impaired healing after photoinduced damage. In addition, vitamin C protects porcine skin from UVA-mediated phototoxic reactions (PUVA) and therefore shows promise as a broad-spectrum photoprotectant.

In a spate of research activity over the past few years, the conclusion that oxygen free radicals are involved in cutaneous sun damage has become well accepted.[1–4] The proposal that the skin's native antioxidant protection system breaks down under this 'photo-onslaught' has been suggested; for example, it has been shown that in response to acute UV exposure, epidermal superoxide dismutase activity declines.[5–6] Glutathione reductase, catalase and reduced glutathione levels in skin have also been shown to decline after only one exposure to UVB radiation;[7] except for glutathione, the same results hold for UVA-visible irradiation of skin.[8] Loss of other skin antioxidants (ubiquiones, vitamins E and C, etc.) ranged from minimal to significant. Prophylactic antioxidant therapy has been investigated in so far as it has been shown that increased dietary antioxidants (including vitamin C) lessen UV-induced skin lesions,[9,10] that topically applied, liposomally encapsulated superoxide dismutase reduces a UV-mediated loss of the native enzyme[11] and that free-radical scavengers inhibit ultraviolet radiation's formation of 'sunburn cells' in excised skin.[12] In addition, topical application of several free-radical scavengers on guinea-pig skin lessened subsequent photosensitized reactions (erythema and oedema).[13] Finally, a recent report has shown topically applied vitamin E or vitamin C effective in moderating low level, chronic UVB (but not UVA) damage to mouse skin.[14]

Vitamin C (L-ascorbic acid) has received renewed attention as an antioxidant of biological importance in systems varying from blood ('. . . an outstanding antioxidant in human blood plasma'[15]) to skin.[16] On the skin surface, it and thioredoxin reductase may be very important in eliminating reasonably long-lived free radicals.[16,17] Although vitamin C is valuable as a free-radical quencher, it may itself be susceptible to UV-mediated destruction. As such, we investigated the ability of pharmacological amounts of topically applied vitamin C to increase cutaneous concentration of this vitamin and to lessen UV damage to porcine skin.

Methods

L-Ascorbic acid (A.C.S. reagent), D-isoascorbic acid, 1,2 propanediol (propylene glycol) and hydroxypropylcellulose (average MW = 300,000) were purchased from Aldrich. Experiments utilized a 10% L-ascorbic acid (w/v) solution in 20% propylene glycol (v/v) with 0·5% hydroxypropylcellulose (w/v) incorporated as thickener. Stored at 4°C, this solution was stable for 1 week as measured spectrophotometrically.

Correspondence: Dr D.Darr, Box 3135, Duke University Medical Center, Durham, NC 27710, U.S.A.

Animals

Domestic Yorkshire pigs (males weighing 32–45 kg) were used in this study because of the similarity of their skin to humans. They were housed in barns at the North Carolina State Veterinary School and fed a standard diet. Prior to experiments, animals were anaesthetized with ketamine/xylazine (2 mg/kg) and their backs clipped with animal shears. During irradiation or before biopsy, all animals were additionally placed under halothane anaesthesia. Typically four animals were used per experiment.

UV source

A bank of two Westinghouse FS40 lamps was used in the bulk of the studies. They were placed above a restrained animal at a distance of approximately 10 cm. The intensity at that distance measured 0·45–0·5 mW/cm^2 using a National Biological Corporation model UVB LMH06C photodetector. Alternatively, specially made Philips TL 40W/01 fluorescent bulbs (four housed in a planar array) were used experimentally. They were placed above the restrained animals to achieve an intensity of $\approx$0·8 mW/cm^2. In the PUVA experiments, four GE F40 BL fluorescent bulbs were used. These were placed above the animals so that the measured intensity was 2 mW/cm^2 (using an International Light Model IL 440 detector). In these experiments, 8-methoxypsoralen was formulated at a 0·1% (w/v) concentration in 90% ethanol. The solution was applied to the sites at 10 μg/cm^2. Additional experimental details are given in the Figure and Table legends.

Percutaneous absorption

Skin was removed from a non-treated animal at the time of killing to a depth of 500 μm with a Padgett Model B dermatome. The skin was cut into 1·5-cm squares and placed between the upper and lower chambers of a modified Franz cell. Vitamin C samples (20 μl) containing 1 μCi ^{14}C-L-ascorbic acid (Amersham) were pipetted on to skin in multiple cells. At 24 and 48 h, 100-μl aliquots were removed from the buffered saline reservoir, mixed with scintillant and counted on an LKB model 1219 scintillation counter. The skin sample itself was then washed by dipping for 10 s into three consecutive water baths (this was shown to remove virtually all surface counts) and burned in a stream of oxygen in a Packard model 306 tissue oxidizer. The $^{14}CO_2$ released was trapped in scintillant and also counted. Combination of the the two values gave an estimate of the penetration of the ascorbic acid into and through the epidermis.

Histology

The experimental sites were biopsied with a 4-mm punch at 24 h for UVB experiments. For UVA experiments, biopsies were taken at 48 h. Like humans,[18] these Yorkshire pigs showed variable, delayed responses to PUVA treatment. The biopsies were fixed in formalin and processed for routine histology. For 'sunburn cell' analysis, duplicate haematoxylin and eosin-stained sections of triplicate biopsies for each experimental site were analysed for sunburn cells (basal keratinocytes having pyknotic nuclei as well as eosinophilic cytoplasm) and normalized to the 4-mm punch diameter. The number of sunburn cells per given condition was calculated in this way from four animals per experiment.

HPLC analysis of vitamin C levels in skin

To determine vitamin C concentrations in the skin, HPLC with electrochemical detection was used.[19] Briefly, after extensive washing, skin was excised at the subcutaneous boundary from two or more animals using a 4-mm punch, and immediately frozen in liquid nitrogen. Weighed skin samples were placed on a glass dish in cold 5% metaphosphoric acid (MPA) and finely minced with surgical scalpels. Following a wash with additional MPA the minced skin sample was subjected to three cycles of freeze-thawing (−70°C). Samples were spun down and the supernatant filtered through a 0·22 μm filter. All samples were frozen at −70°C until analysis.

Laser-Doppler measurement of cutaneous blood flow

Laser-Doppler velocimetry (LDV) is an accepted non-invasive technique for monitoring relative blood flow and has been used in quantifying erythema caused by UV irradiation.[20] Cutaneous blood flow was estimated by LDV using a LaserFlo™ BPM model 403 24 h after UV irradiation of vitamin C or vehicle-treated sites on four animals.

Results

Skin levels of vitamin C after topical treatment with this vitamin

Using modified Franz cells, percutaneous absorption studies with ^{14}C-labelled ascorbic acid showed that

Table 1. Increase in cutaneous vitamin C levels by topical treatment with this vitamin. Four-millimetre biopsies from each treatment site on each of three animals were weighed, pooled and extracted as in Methods. Samples were analysed in duplicate by HPLC with electrochemical detection and peak areas compared to known standards. Values are mean ± SE of duplicate analyses of two pooled samples.

Site	Vitamin C concentration μg/g wet weight
Vehicle-treated	20·2 ± 1·9
Vitamin C-treated	542·6 ± 50·1*

* $P<0.005$, Student's *t*-test.

0·7 ± 0·1% (mean ± SEM) passed through and 8·2 ± 1·1% (mean ± SEM) of the applied dose (2 mg) was present within a 500-μm thick section of porcine skin 48 h after topical application. However, this technique only follows the movement of the radioactive label and as vitamin C is notoriously unstable, we also measured cutaneous levels of vitamin C proper by HPLC. Table 1 is derived from an experiment in which three animals were treated topically with vitamin C, then thoroughly washed, and full thickness skin samples excised and assayed for ascorbic acid levels. Additional experiments with different animals have confirmed that skin levels of vitamin C increase 4–40-fold or more following multiple treatments with this vitamin. Assuming porcine skin to be approximately 75% water (a fair approximation), both methods give values of vitamin C concentration in the skin of approximately 3–4 mM after topical application. Because of the obvious gradient, even higher levels of this vitamin can be assumed in the upper levels of the epidermis and stratum corneum while somewhat lower in the reticular dermis.

Protection of swine skin from UVB by topical vitamin C

Topical application of vitamin C protects porcine skin from UVB damage. Using sunburn cell (dyskeratotic basal epidermal cells) formation as a marker for UV-induced damage, Table 2 shows a significant reduction in the UV-mediated formation of this cell type by topical application of vitamin C. Although a single application (approximately 15–30 min prior to exposure) is generally sufficient to give UV protection, some animals showed better results if the vitamin C was applied more than once, and as such, a typical protocol called for several applications of the vitamin to the skin prior to irradiation.

Table 2. Reduction in UVB-mediated sunburn cell formation by topical vitamin C. Paired sites on the backs of domestic pigs were pretreated for 3 days, and 30 min before irradiation, with a 10% vitamin C solution or the vehicle control (100 μl/10 cm^2). The sites were then irradiated with the indicated dose of UVB from a bank of Philips TL 40W/01 bulbs At 24 h, three biopsies were taken from each site. Duplicate sections from each biopsy were stained with haematoxylin and eosin and the number of 'sunburn cells' counted. Cell counts from the six histological sections per condition were normalized to the 4-mm biopsy diameter. Values are the mean ± SE. Numbers in parentheses equal animal number. From this UV source in these animals 100 mJ/cm^2 ≈ 1 MED

Condition	No. of sunburn cells/4-mm biopsy (dose 400 mJ/cm^2)
Control (12)	33·1 ± 5·1
Vitamin C-treated (12)	20·5 ± 3·0*

* $P<0.005$; paired *t*-test.

The protection noted in Table 2 is not, however, due to a 'sunscreen effect'. The UV source in those experiments was a bank of four Philips TL 40W/01 bulbs whose emission centres around 311 nm with little or no output below 305 nm (Fig. 1). The L-ascorbic acid in this formulation has virtually no absorbance above 300 nm. Indeed, if this solution is applied to a piece of quartz glass in a similar manner to that employed experimentally (≈100 μl/10 cm^2) and allowed to air dry for 30–60 min, virtually 100% of the energy at wavelengths greater than 300 nm is transmitted through the quartz cuvette. Therefore, the protection seen against UVB is biological, not physical. Responses to these Philips bulbs (sunburn cell formation and erythema) were indistinguishable from those seen with more typical UVB bulbs, e.g. Westinghouse FS40.

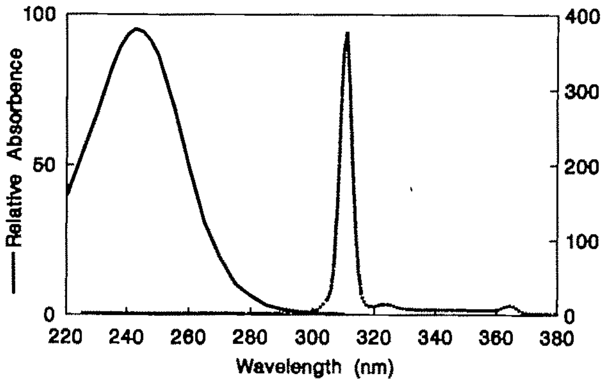

Figure 1. Absorption spectrum of L-ascorbic acid versus emission spectrum of Philips TL 40W/01 fluorescent bulbs. The absorbence of the vitamin C in the test solution was measured through quartz cuvettes using a Shimadzu Model 260 spectrophotometer. The emission spectral data was obtained from the manufacturer. (—) Relative absorbence of L-ascorbic acid in experimental formulation. (----) Emission spectrum of TL 40W/01 fluorescent bulb.

Table 3. Reduction in UVB-elicited erythema by topical vitamin C. Paired sites on the backs of domestic pigs were pretreated for 1 week, and 30 min before irradiation, with a 10% vitamin C solution or the vehicle control. The sites were then irradiated with approximately 2–3 MED of UVB radiation (≈150 mJ/cm² from two Westinghouse FS40 bulbs) At 24 h, triplicate measures of cutaneous blood flow from each site were taken using a TSI LaserFlo™ model 403 laser-Doppler velocimeter. Adjacent non-irradiated skin was used for 'background' blood flow determination. Values are mean ± SE of arbitrary blood flow values after subtraction of 'background' flow. Numbers in parentheses equal animal number

Condition	Cutaneous blood flow
Control (4)	2·91±0·50
Vitamin C-treated (4)	1·40±0·70*

* $P<0·001$, paired *t*-test.

A second hallmark of UVB-induced sunburn is erythema. One quantitative measure of erythema is provided by laser-Doppler velocimetry. Using this technique, increased skin blood flow occurred 24 h after a 2-3 MED dose of UVB was measured. In this experiment, blood flow approximately doubled compared with adjacent non-irradiated control sites in response to the radiation. Pretreatment with topical vitamin C halved this increase (Table 3).

Protection against UVB damage by reducing and non-reducing analogues of vitamin C

The antioxidant protection of skin by vitamin C should be mimicked by its isomer isoascorbic acid, assuming cellular uptake and/or *in vivo* penetration rates are similar for the two molecules (not an absolute, e.g. Kipp and Schwartz[21]). Table 4 shows that both ascorbic acid and isoascorbic acid exhibit identical protective abilities against UVB damage. In a separate experiment, the two-electron oxidation product of ascorbic acid, dehydroascorbate (DHA), provided little or no visual protection against UV erythema when compared with its parent molecule (data not shown).

Table 4. Comparative effectiveness of ascorbic acid vs. isoascorbic acid in preventing UVB-induced 'sunburn cell' formation. Solutions were 10% (w/v) in 20% propylene glycol; 1 0% hydroxy-propylcellulose. Paired sites on the backs of domestic pigs were treated daily for 1 week, and 30 min before irradiation, with 2–3 MED of UVB (Westinghouse FS40 bulbs). After 24 h, 4 mm biopsies (three per site) were taken, processed for histology, stained with haematoxylin and eosin and 'sunburn cells' counted as in Table 2. Numbers in parentheses equal animal number.

Treatment	No. of sunburn cells/4 mm	% Reduction
Vehicle (8)	17·2±6·0	
L-ascorbic acid (8)	10·0±3·7*	42
Vehicle (8)	18·3±4·5	
isoascorbic acid (8)	10·8±2·5*	41

* $P<0·02$; paired *t*-test.

Protection of pig skin from UVA-mediated phototoxicity by topical vitamin C

Although nowhere near as erythemogenic or carcinogenic as UVB, UVA is responsible for many biological effects, at least partially because the relative intensity of UVA in solar UV is many times that of UVB. Because of the aforementioned ineffectiveness of low-dose UVA to elicit measurable biological responses in skin, we chose to use 8-methoxypsoralen plus UVA (PUVA) to increase skin damage, i.e. sunburn cell formation, and allow us to quantify possible protection by topical vitamin C. This method has been used to test UVA sunscreen efficacy, and to assess the role of reactive oxygen in PUVA damage *in vivo*.[13,22–24] Table 5 shows that topical application of vitamin C does inhibit the PUVA-mediated production of this cell type, indicating that vitamin C is capable of ameliorating a UVA-mediated phototoxic response in skin. Indeed, in experiments using UVA doses greater than 500 mJ/cm², gross pathological changes (leucocytic infiltrates, blistering, etc.) are seen

Table 5. Reduction in psoralen-UVA-mediated sunburn cell formation by topical vitamin C. Paired sites on the backs of domestic pigs were pretreated for 3 days with a 10% vitamin C solution or the vehicle control. One hour prior to irradiation, 8-methoxypsoralen (0·1% w/v in 90% ethanol) was evenly applied to all sites (10 μg/cm²). Thirty minutes later a final treatment of vitamin C or vehicle (100 μl) was applied The sites were then irradiated with a bank of GE F40 BL fluorescent tubes (emission peak ≈360 nm) at the indicated dose. In these animals, this is approximately equal to three times the minimum phototoxic dose (MPD). Biopsies were taken at 48 h. Sunburn cell analysis was performed as in Table 2. Numbers in parentheses equal animal number

Condition	No. of sunburn cells/4-mm biopsy (dose 500 mJ/cm²)
Control (10)	114·5±17·8
Vitamin C-treated (10)	48·4±10·1*

* $P<0·002$; Paired *t*-test.

Table 6. UV-mediated loss of cutaneous vitamin C. Sites on three animals were treated with topical vitamin C (daily for 5 days). One site was then covered and the other exposed to approximately 4 MED of UVB. Immediately after the exposure, the sites were thoroughly washed. Multiple biopsies were taken from the two sites on each animal, frozen in liquid nitrogen and stored at − 70°C until analysed. In two separate analyses, biopsies from the irradiated or non-irradiated sites from the three animals were pooled, weighed and extracted for HPLC quantification of vitamin C content. Vitamin C-treated, non-irradiated values were treated as 100% and irradiated values compared with them. Numbers represent the average of five HPLC determinations per experimental condition.

Treatment	% Vitamin C remaining
Vitamin C-treated	
Non-irradiated	100
Irradiated	34

in vehicle-treated sites, whereas vitamin C pretreated sites maintained substantially normal histology (unpublished data). No evidence was found for any interaction between vitamin C and the ground-state psoralen molecule (measured spectrophotometrically) so the protection is presumed to be due to either quenching of the excited state psoralen molecule by vitamin C or its scavenging of reactive oxygen species known to be generated during PUVA therapy.[13]

Loss of cutaneous vitamin C by UV radiation

Vitamin C is essential to tissue repair, a fact noted many years ago. We, and others,[5–7] hypothesized that UV damage may in part be due to a depletion of the skin's natural defences by the radiation. In fact, Fuchs *et al.*[7] have shown UV-mediated losses of several key antioxidants in mice exposed to UVB. Vitamin C levels (measured spectrophotometrically) declined, although not significantly. We found it difficult to document ultraviolet radiation-induced changes in vitamin C concentration in full thickness skin; vasodilatation and skin inflammation may lead to variable levels. To circumvent this problem, we used several days of topical vitamin C treatment to raise skin levels of the vitamin. The treated areas were then UVB-irradiated, washed thoroughly to remove any free vitamin C, and the cutaneous vitamin C concentration measured by HPLC. Table 6 shows a dramatic UV-mediated diminution of the tissue vitamin C. It thus appears likely that the natural vitamin C levels of the skin are also subjected to this loss, perhaps to near depletion in areas.

Discussion

Although the mechanism(s) of ultraviolet damage to skin is certain to be complex, reactive oxygen species are likely to be a major contributor to this damage. Unlike model systems, UV probably generates the entire spectrum of oxygen radicals, radical-derived species and other reactive molecules. In this regard, ascorbic acid should be particularly effective in interfering with the UV-mediated generation and/or propagation of the reactive oxygen species, as it reacts with or quenches the superoxide anion,[25] the hydroxyl radical,[26] singlet oxygen[27] and hypochlorous acid.[28] In addition, vitamin C is the primary replenisher of vitamin E, the pre-eminent inhibitor of lipid peroxidation.[29,30] Durham *et al.* showed that increases in dietary vitamin C led to a statistically significant reduction in subsequent UV-elicited tumours.[10] We believed that topical application would achieve a much higher tissue concentration of this vitamin compared with oral intake. Experiments are in progress to specifically test this. However, conservative calculations based on the percutaneous absorption and HPLC experiments give ascorbic acid concentrations in the millimolar range in full or partial thickness skin after several days of topical application. These levels would be expected to be even higher in the superficial layers of the skin, i.e. the epidermis and upper papillary dermis. *In vitro* evidence suggests that at these concentrations ascorbic acid strictly acts as an antioxidant.[31] Experiments with the isoascorbate and dehydroascorbate also show the effects to be biological and dependent on the reducing property of this vitamin. It is of note that, although it is known that dehydroascorbate can be metabolically converted back to the parent ascorbic acid molecule within cells, this apparently does not occur under our experimental conditions, or more likely, the bulk of the protection occurs in the metabolically inactive cell layers, i.e. the stratum corneum. In defense of this thesis, using vitamin C solutions at either pH 2·5 (totally un-ionized) or 5·0 (totally ionized) makes no difference in protection against UVB damage even though the un-ionized form traverses the skin to a much greater degree (data not shown). In addition, as previously mentioned, protection is noted after only one application of the vitamin, 15–30 min before UV exposure, presumably long before dehydroascorbate could reach the metabolically active layers of the epidermis. Thus, it is not unexpected for DHA to be inactive in this model and reasserts the importance of the reductant nature of vitamin C in the lessening of damage noted.

These studies in part employed fluorescent UVB-emitting bulbs, wavelengths long known to be responsible for eliciting skin cancer. Multiple exposures to significantly lower doses than used in this study has been shown to be carcinogenic.[32] Additionally, reactive oxygen species/oxygen radicals have been implicated in the tumour promotion phase.[32,33] One study has shown a positive effect of topical vitamin C in preventing the onset of tumourogenesis in mice.[14] This is an ongoing avenue of study.

Quantitative measures (sunburn cell counts and laser-Doppler measurements) show that topical vitamin C treatment can reduce UVB-mediated increases in these parameters ≈40% at moderate UV doses (3–4 MED). Tissue hypoxia has also been shown to reduce UVB-induced formation of suburn cells, again by approximately 40–50%.[34] These data suggest that, although important, reactive oxygen species cannot be considered the only source of UVB damage. Certainly, historical experiments showing direct (non-oxygen-dependent) interaction between UV (particularly UVB) and important biological targets, e.g. DNA, are well known. Vitamin C obviously need not interfere with these other reactions.

Recent studies hint at a potential drawback to many ordinary sunscreens. They absorb the sunburning rays of the sun so effectively that they allow the user to stay outdoors much longer than would ordinarily be possible. Unfortunately, this allows considerable amounts of the longer wavelength, non-absorbed UVA to enter the skin, which may be related to an increased risk of certain skin cancers or ageing changes. In addition, there appears to be a stronger correlation between UVA-induced skin damage and oxygen radical formation than with UVB.[35] As we have now shown, topical vitamin C is also effective in moderating UVA-mediated phototoxic reactions in skin, apparently due to its antioxidant status. Earlier *in vitro* work showed that ascorbic acid and anaerobiosis were equally effective in inhibiting PUVA-mediated intermolecular cross-link formation of α-crystallin, providing further evidence for the participation of reactive oxygen species in at least some psoralen-UVA photosensitized reactions.[36] These results are in contrast with investigations performed in mice, i.e. vitamin C was ineffective in preventing UVA-induced skin wrinkling.[14] Considerable differences, e.g. long-term UVA exposure vs. acute PUVA treatment, mouse vs. pig skin, as well as measured endpoints, exist between these two studies. Further studies will be necessary to understand the reasons for these differences. Whether the mechanisms responsible for PUVA damage in skin are the same as those leading to chronic UVA damage is a subject for debate. As there are no good models for UVA damage alone, PUVA has become an acceptable experimental model for predicting product efficacy in providing protection against longer wavelength ultraviolet radiation (UVA).[23,24] Topical vitamin C treatment may thus prove to be a particularly effective broad spectrum photoprotectant.

Participation of reactive oxygen species in, and antioxidant prevention of, UV damage is now well documented.[1–4,13,14,37] We report that topically applied L-ascorbic acid is effective in preventing UVB- and psoralen-UVA-mediated damage to pig skin. The mechanism(s) of this protection is presumed to be due to its 'antioxidant' status. The protection may be due to vitamin C directly reacting with, or quenching certain reactive species, or by regenerating vitamin E and thus participating in the inhibition of UV-induced lipid peroxidation. As significant free-radical reducing potential is found in the extracellular spaces,[16] topical vitamin C may significantly bolster this free-radical reducing capacity and thus benefit the skin. The skin's innate antioxidant defense system is complex. We believe that vitamin C is an important part of the UV defense system of the skin, although its exact role has not yet been fully elucidated.

Finally, vitamin C is essential to tissue and/or wound repair. Perhaps UV damages skin not only directly, but indirectly via inactivation of protective agents such as superoxide dismutase, catalase, etc. We provide evidence that additionally, skin vitamin C levels can be dramatically reduced in response to UV radiation. Thus, in addition to a loss of antioxidant status, it may be hypothesized that other tissue damage may be less effectively repaired in this 'vitamin deficient' state. Therefore, replenishment of skin vitamin C would be an important pharmacological intervention against sun damage.

Acknowledgments

This work was supported in part by the Dermatology Foundation (Dermik, Inc.), the Skin Cancer Foundation and NIH Grants 5R37 AR17128 and 5R01 AR28304.

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British Journal of Dermatology (1992) 127, 254–257.

Increased levels of inflammatory cytokines in human skin lymph derived from sodium lauryl sulphate-induced contact dermatitis

T.HUNZIKER, C.U.BRAND, A.KAPP,* E.R.WAELTI† AND L.R.BRAATHEN

Dermatological Clinic and †Institute of Pathology, University of Berne, Switzerland

**Department of Dermatology, University of Freiburg, Germany*

Accepted for publication 10 March 1992

Summary

A superficial peripheral lymph vessel draining the skin of the upper and medial part of the foot was cannulated on the lower leg of six healthy human volunteers. After 2 days an irritant contact dermatitis was induced by application of 10% sodium lauryl sulphate to the area of skin drained by the lymph vessel. Three days later the spontaneously regressing skin reaction was treated with clobetasol propionate. Lymph was collected twice daily for 7 days, and the levels of various cytokines (IL-1α, IL-1β, IL-2 and soluble IL-2 receptors, IL-6, IL-8, TNF-α, GM-CSF) were determined by ELISA technique. In the majority of the volunteers all cytokines examined were detected in several lymph samples, with the exception of IL-1α and IL-8. In parallel with the clinical symptoms of the contact dermatitis the levels of IL-6 and TNF-α increased 8–10-fold, whereas for IL-1β, IL-2, IL-2 receptors, and GM-CSF there was a delayed, 2–3-fold increase. These results suggest that cytokines, in particular IL-6 and TNF-α, may actively participate in the immunological reactions in the skin and in the regional lymph nodes during contact dermatitis.

In recent years substantial data has been obtained concerning immunological processes in the skin, whereas the human skin-derived lymph still represents a missing link between the reactions in the skin and those in the regional lymph nodes. Cytokines are produced by a variety of cells in the skin and play an important role in inflammatory reactions.[1,2] We recently described a technique to obtain human skin-derived lymph[3] and now report that an irritant contact dermatitis induces significant changes of cytokine levels in the afferent skin lymph.

Methods

Lymph sampling

In six healthy volunteers a superficial peripheral lymph vessel was cannulated on the antero-medial aspect of one leg as previously described.[3] Lymph vessels in this area drain the skin of the upper and medial part of the foot. Because there are no anastomoses between the subfascial and the epifascial lymphatic trunks in this region, their lymph is essentially skin-derived. For 7 days, the lymph was collected in sterile plastic vials (Greiner, Langenthal, Switzerland) containing 1 ml of 0·9% NaCl with 20 IU heparin. By changing the vials at 8 a.m. and 5 p.m., we distinguished between day and night sampling periods. After measuring the volume and separating the cells by centrifugation at 300 *g* for 5 min, the lymph samples were snap-frozen and stored at −70°C. Selected lymph samples of up to 200 μl were also harvested by gentle massage and snap-frozen immediately. Protein detection in the lymph samples was performed using the Coomassie Plus protein assay reagent (Pierce, Rockford, IL, U.S.A.).

Induction of irritant contact dermatitis

On the third and fourth days after cannulation, 10% sodium lauryl sulphate (SLS) in aqueous solution was applied under occlusion to the skin of the drained area on the upper and medial part of the foot. On days 6 and 7 clobetasol propionate 0·05% cream (Dermovate®) was applied three times to the spontaneously regressing skin reaction. The skin reaction was evaluated twice daily and quantified by visual scoring as: 0, no response; 0·5, slight, patchy erythema; 1, uniform erythema; 2, ery-

Correspondence: Dr T.Hunziker, Dermatological Clinic, Inselspital, 3010 Berne, Switzerland.

thema and oedema; 3, erythema, oedema and vesiculation; 4, superficial necrosis.

Cytokine analysis

With the exception of IL-8, cytokine concentrations in all lymph samples were determined using commercially available ELISA kits. Quantikine ELISA kits (R&D systems/Biermann Diagnostica, Bad Nauheim, Germany) were used for measurement of human IL-1α, IL-1β, IL-2, IL-6 and TNF-α. Soluble IL-2 receptors were detected using the Cellfree IL-2 Receptor test kit (T cell sciences/Biermann Diagnostica, Bad Nauheim, Germany). GM-CSF concentrations were determined by the Factor Test GM-CSF ELISA kit (Genzyme/IC Chemikalien, München, Germany). IL-8 was measured by a solid-phase double-ligand ELISA method as described previously.[4] The biological activity of IL-6 was determined on IL-6-dependent hybridoma cells (mouse-mouse hybrid 7TD1), as described by Van Snick *et al.*[5] Briefly, serial dilutions of the test samples were incubated with 2×10^3 7TD1 cells at 37°C for 4 days in microtitre plates. Cell densities were then determined by colorimetric measurement of the hexosaminidase levels.[6]

Results

The mean scores of the inflammatory skin response to 10% SLS are shown as background in Figures 1–6. All volunteers developed a mild to moderate erythema and some also slight oedema, with a peak reaction 2 days after the first application of the irritant. The reaction then faded spontaneously, and after application of clobetasol propionate disappeared within 36 h. Data on lymph flow and output of cells of the six volunteers have been reported previously.[3]

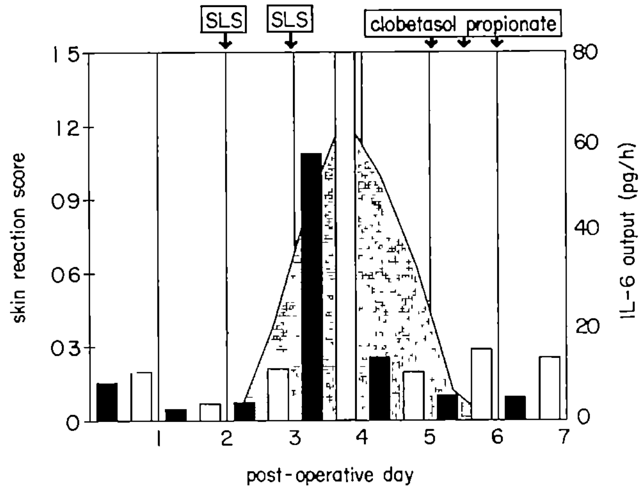

Figure 2. The mean scores of the skin reaction and the mean output of IL-6 before and after application of sodium lauryl sulphate followed by clobetasol propionate ($n=4$). ▨, skin reaction; ☐, day; ■, night.

The protein content of the lymph samples (Fig. 1) was closely correlated initially with the surgical procedure of the lymph vessel cannulation and later with the SLS-induced inflammatory skin reaction. Similar to lymph flow and output of cells,[3,7] the day values exceeded those of the night.

The levels of the cytokine output varied substantially between the volunteers, which might at least in part be due to varying topography and calibre of the cannulated

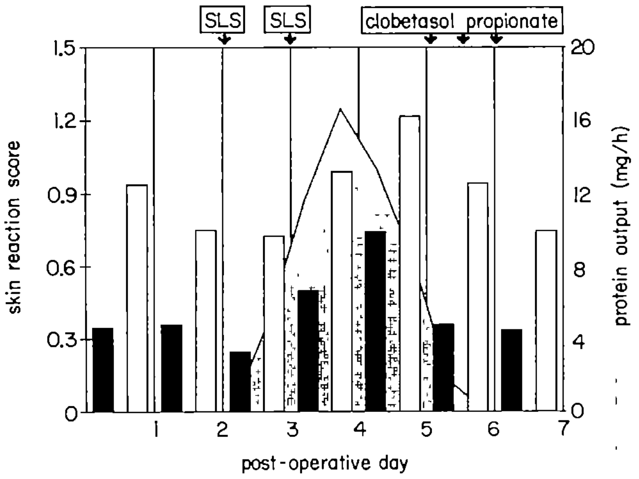

Figure 1. The mean scores of the skin reaction and the mean output of protein before and after application of sodium lauryl sulphate followed by clobetasol propionate ($n=4$). ▨, skin reaction; ☐, day; ■, night.

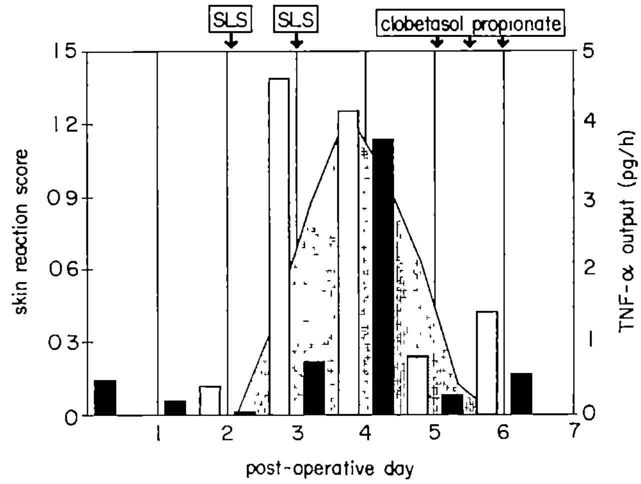

Figure 3. The mean scores of the skin reaction and the mean output of TNF-α before and after application of sodium lauryl sulphate followed by clobetasol propionate ($n=4$). ▨, skin reaction; ☐, day; ■, night.

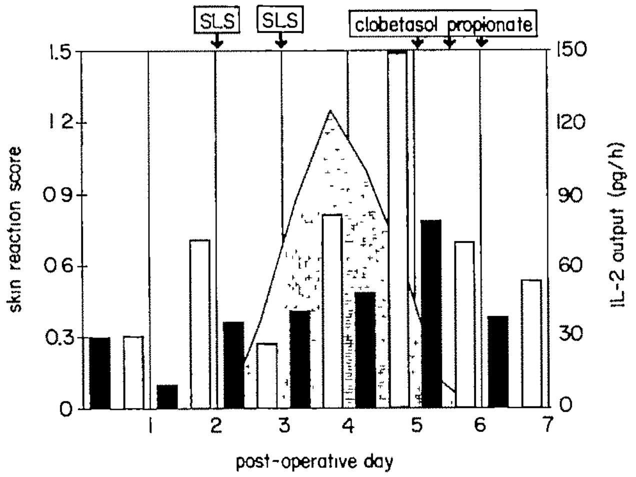

Figure 4. The mean scores of the skin reaction and the mean output of IL-2 before and after application of sodium lauryl sulphate followed by clobetasol propionate ($n=4$). ▨, skin reaction; □, day; ■, night.

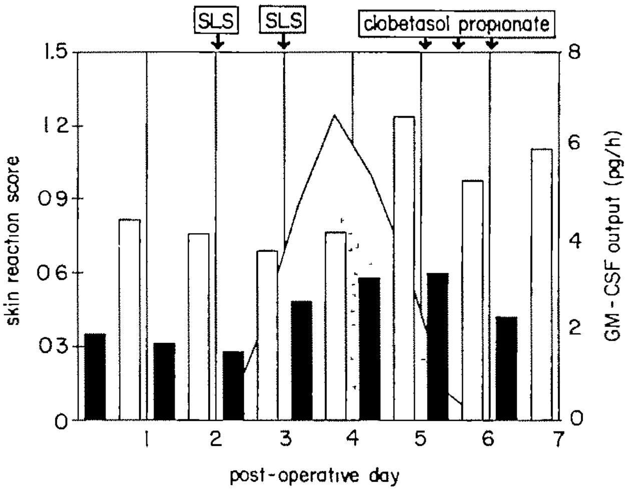

Figure 6. The mean scores of the skin reaction and the mean output of GM-CSF before and after application of sodium lauryl sulphate followed by clobetasol propionate ($n=4$). ▨, skin reaction; □, day, ■, night.

lymph vessels.[3] The respective time-courses, however, exhibited little inter-individual variation, maximum outputs of a given cytokine always occurring within a common 24-h period.

Because of technical problems[3] two of the volunteers could not be included in the complete time-course analysis of cytokine output. In the remaining four volunteers IL-6 (Fig. 2) and TNF-α (Fig. 3) exhibited an 8–10-fold increase during the early phase of the contact dermatitis, compared with the low values in the post-operative period. The values of IL-6 exceeded those of TNF-α more than 10-fold. Parallel with fading of the skin reaction, the output of IL-6 and TNF-α then rapidly dropped to near-baseline values. A different pattern of output in the lymph was encountered for IL-1β (not shown), IL-2 (Fig. 4), IL-2 receptors (Fig. 5), and GM-CSF (Fig. 6). Although there was an increase after induction of the contact dermatitis, values were at most 2–3-fold those of the baseline, and they were reached either in the late phase, or after clinical resolution, of the skin reaction. Values of IL-2 far exceeded those of IL-1β (mean below 10 pg/h) and GM-CSF. As a rule, night values were again lower than corresponding day values. Low levels of IL-1α and IL-8 were detected sporadically, and there was no correlation with the skin reaction (not shown).

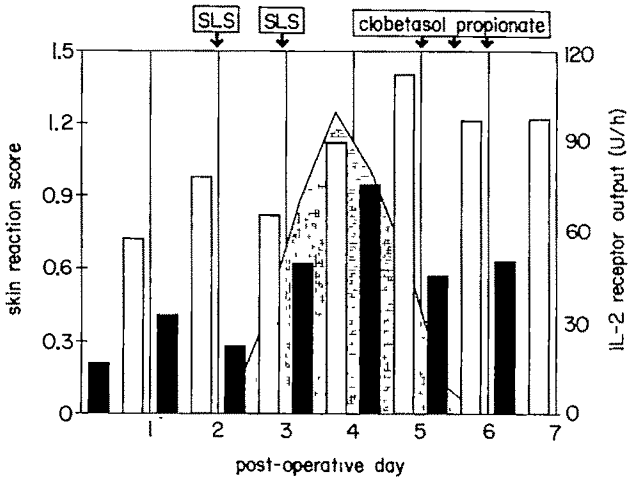

Figure 5. The mean scores of the skin reaction and the mean output of soluble IL-2 receptors before and after application of sodium lauryl sulphate followed by clobetasol propionate ($n=4$). ▨, skin reaction; □, day; ■, night.

In a freshly harvested and immediately snap-frozen morning lymph sample from one volunteer the IL-6 value was 15 pg/ml. In contrast, the value of IL-6 in the lymph of the previous night's sampling period was 93 pg/ml. Both samples derived from the phase of established skin reaction. In lymph samples of two volunteers also derived from this phase of the experiment, the levels of biological activity of IL-6 corresponded with those in the ELISA assay (not shown).

Discussion

In this human *in vivo* system to investigate afferent skin lymph we demonstrated significant increases of IL-6 and TNF-α in the early phase of an SLS-induced irritant contact dermatitis. A major part of these inflammatory cytokines is probably released during the 9-h (day) and 15-h (night) lymph sampling periods, as demonstrated for IL-6. During these periods various inflammatory cells (Langerhans cells, monocytes, T and B lymphocytes [publication in preparation]) are 'incubated' in the lymph, and are probably exposed to a variety of immunomodulating mediators derived from a number of different cell types (keratinocytes, Langerhans cells, monocytes, T and B lymphocytes, granulocytes) involved in the inflammatory skin reaction. Apart from cytokines, mediators of the arachidonic acid and the complement cascade must be considered. At present, the wide range of possible complex interactions prohibits detailed interpretation. However, increased levels of the two multifunctional cytokines IL-6 and TNF-α in the skin lymph derived from the early phase of an irritant contact dermatitis may represent a specific sign of the cutaneous inflammatory response, suggesting a primary function in the local pathomechanisms, and also points to a possible role in the regulation of the immunological response in the regional lymph nodes. Recently, increased keratinocyte expression of IL-6, but not of TNF-α, has been reported in allergic and irritant patch-test reactions.[8] Other multifunctional cytokines such as IL-1 and IL-8 did not show a relevant, dermatitis-associated increase, which lends further support to this concept. These findings might result from a specific monocyte activation during the early phase of an irritant contact dermatitis. Alternatively, IL-6 and TNF-α could also be released by 'activated' keratinocytes. These hypotheses are further supported by the finding that the output of T-cell-dependent mediators, such as IL-2 and its receptor as well as of GM-CSF, does not increase impressively during the early phase of the skin reaction. Moderate increases in the late phase, or even after resolution, of the contact dermatitis may in part correspond to a wash-out of mediators secondarily released in the course of the inflammatory skin reaction.

While it is very likely that cytokines are an important element of pathogenic mechanisms in inflammatory skin diseases,[1,2] present knowledge of the complex interactions of cytokines and cellular targets does not allow formulation of hypotheses concerning a specific role in the pathomechanisms of a defined skin disease. However, the data presented clearly indicate that local inflammation may lead to a significant increase of biologically active, probably monocyte- or keratinocyte-derived inflammatory cytokines in the afferent skin lymph. Therefore it appears likely, that local inflammation may induce systemic inflammatory responses via cytokines transported to the regional lymph nodes. We hope that analysis of the cell types present in the lymph, of other types of mediators, and also of adhesion molecules expressed by the lymph-derived cells will result in an improved understanding of the inflammatory processes during an irritant contact dermatitis.

Acknowledgments

We thank Dr B.Dewald, Theodor-Kocher-Institute, University of Berne, for the measurements of IL-8, and Dr D.Muller, Institute of Pathology, University of Berne, who determined the biological activity of IL-6. This work was supported by a grant from the Swiss National Fund (23-7774.89).

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British Journal of Dermatology (1992) 127, 258–261.

Differential effect of griseofulvin on interferon-γ-induced HLA-DR and intercellular adhesion molecule-1 expression of human keratinocytes

K.TAMAKI, A.SAITOH AND N.YASAKA

Department of Dermatology, Yamanashi Medical College, 1110 Shimokatoh, Tamaho-cho, Nakakoma-gun, Yamanashi-ken 409-38, Japan

Accepted for publication 5 May 1992

Summary Interferon-γ (IFN-γ) induces major histocompatibility complex (MHC) class II antigen and intercellular adhesion molecule-1 (ICAM-1) expression on human epidermal keratinocytes. Griseofulvin, an extensively used antifungal agent, has been used effectively in the treatment of lichen planus, in which keratinocytes express both HLA-DR and ICAM-1 antigens. In this study, we investigated the effect of griseofulvin on IFN-γ-induced MHC class II antigen and ICAM-1 expression of keratinocytes, and demonstrated that griseofulvin decreased IFN-γ-induced HLA-DR but not ICAM-1 expression.

It is well established that interferon-γ (IFN-γ) induces major histocompatibility complex (MHC) class II antigen (Ag) and intercellular adhesion molecule-1 (ICAM-1) expression on human keratinocytes.[1,2] It is also known that HLA-DR and ICAM-1 expression on keratinocytes is differentially regulated.[3]

HLA-DR and ICAM-1 expression on keratinocytes has been demonstrated in various skin diseases such as lichen planus, allergic contact dermatitis and psoriasis vulgaris.[4,5] Thus, it would be reasonable to speculate that the modulation of these molecules may affect the disease state. In this context, in a previous paper we have shown that 1,25-dihydroxyvitamin D_3, the most active metabolite of vitamin D, now used for the treatment of psoriasis vulgaris,[6] decreased IFN-γ-induced HLA-DR expression but not ICAM-1 expression on keratinocytes.[7] This could be one mechanism contributing to the effectiveness of 1,25-dihydroxyvitamin D_3 in psoriasis vulgaris.

Griseofulvin was first used for the treatment of lichen planus by Sehgal *et al.* in 1971.[8] Subsequent investigators confirmed its effectiveness in this disorder.[9–11] Its mechanism of action, however, is unknown.

In this study, we investigated the effect of griseofulvin on IFN-γ-induced HLA-DR and ICAM-1 expression on human keratinocytes. The results show that griseofulvin decreases IFN-γ-induced HLA-DR but not ICAM-1 expression.

Methods

Cell culture

Human keratinocytes were obtained from Sanko Junyaku as Epipack (Clonetics Co., San Diego, CA, U.S.A.) and used at the third passage. Cells were maintained in serum-free keratinocyte growth medium (KGM; Clonetics Co.). KGM consists of MCDB 153 medium plus added growth factors: epidermal growth factor (EGF, 10 ng/ml), insulin (5 μg/ml), hydrocortisone (0·5 μg/ml), and bovine pituitary extract protein (BPE, 70 ng/ml).[12]

Human IFN-γ and griseofulvin

Recombinant IFN-γ was purchased from Collaborating Research Incorporated, Two Oak Park, Bedford, MA, U.S.A. and griseofulvin was a gift from Glaxo Pharmaceutical Co. Ltd., Japan.

Serological reagents

The primary monoclonal antibodies (MAb) used in this study are listed in Table 1. These antibodies were used at a dilution of 1:100. To visualize, FITC-conjugated goat anti-mouse IgG (F(ab′)$_2$ fragment) [Becton Dickinson] was used at a titre of 1:20.

MHC class II Ag or ICAM-1 induction on keratinocytes

Keratinocytes were subcultured into each well of 24-

Correspondence: Dr K.Tamaki.

Table 1. Primary monoclonal antibodies used

MAb	Clone	Isotype	Source*
HLA-DR	L243	IgG2a	BD
Leu 10 (HLA-DQ)	SK10	IgG1	BD
HLA-DP	B7/21	IgG1	BD
ICAM-1	84H10	IgG1	BD
Leu 2a	SK1	IgG1	BD
Leu 2b	SKQ	IgG2a	BD

* BD, Becton-Dickinson.

well collagen-coated plates (Corning). These keratinocytes were cultured with either IFN-γ or griseofulvin, or a combination of IFN-γ and griseofulvin at 37°C in 5% CO_2 for 48 h or 72 h. They were then trypsinized with 0·5% trypsin (Type XI, Sigma, St Louis, MO, U.S.A.) and then washed three times with RPMI 1640, supplemented with 10% fetal calf serum (FCS). Keratinocytes were then stained for MHC class II Ag or ICAM-1.

Immunofluorescence procedure

Keratinocytes were incubated with anti-HLA-DR, anti-HLA-DQ, anti-HLA-DP, or anti-ICAM-1 MAb for 30 min at 4°C. After washing three times with supplemented RPMI 1640, these cells were then incubated with 1:20 diluted $F(ab')_2$ fragment of FITC-conjugated goat anti-mouse IgG at 4°C for 60 min and washed three times with supplemented RPMI 1640. The cells were then examined for cell surface fluorescence under petroleum jelly-lined coverslips according to the method described previously.[13] Anti-Leu 2a antibody and Leu 2b antibody were used as controls.

Viability

The viability of keratinocyte suspensions after various treatments was checked by the trypan blue dye exclusion test.

Statistical analysis

Experiments were performed at least three times and representative results are presented. At least 400 cells were counted in triplicate and results are expressed as the arithmetic mean value±standard deviation. Student's *t*-test was used and a P value of $<0·05$ was considered to be significant.

Results

Induction of HLA-DR, -DQ, -DP and ICAM-1 by IFN-γ

Keratinocytes were incubated with varying amounts of IFN-γ (100 and 400 U/ml) for 48 or 72 h. As shown in Table 2, at 48 h IFN-γ-induced HLA-DR on 10·0% and 29·4% of keratinocytes when cells were incubated with 100 U/ml or 400 U/ml of IFN-γ, respectively. At 72 h, HLA-DR expression was noted on 40·9% and 73·2% of cells when incubated with 100 U/ml or 400 U/ml respectively. Keratinocytes did not express HLA-DQ or -DP even after 72 h incubation.

For ICAM-1 expression, 22·5% or 49·6% of cells were positive when incubated with 100 U/ml or 400 U/ml of IFN-γ for 48 h, respectively. At 72 h, ICAM-1 expression was noted on 59·8% and 67·3% of cells when incubated with 100 U/ml or 400 U/ml of IFN-γ, respectively.

Effect of griseofulvin on IFN-γ-induced HLA-DR expression

To investigate the effect of griseofulvin on IFN-γ-induced HLA-DR, -DQ or -DP expression, IFN-γ (400 U/ml) and/or griseofulvin (1, 4 and 10 μg/ml) were added to keratinocytes and cultured for 72 h. Representative data

Table 2. Percentage of HLA-DR, and ICAM-1 positive keratinocytes after incubation with IFN-γ

IFN-γ (U/ml)	HLA-DR		HLA-DQ		HLA-DP		ICAM-1	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
0	0	0	0	0	0	0	0	0
100	10·0±2·5	40·9±1·2	0	0	0	0	22·5±3·1	59·8±7·3
400	29·4±1·3	73·2±4·2	0	0	0	0	49·6±4·1	67·3±3·8

Keratinocytes were incubated with varying amounts of IFN-γ for 48 or 72 h. HLA-DR or ICAM-1 positive cells were counted after visualization with indirect immunofluorescence. Values are means±SD.

Table 3. Percentage of HLA-DR, and ICAM-1 positive keratinocytes after incubation with IFN-γ and/or griseofulvin

IFN-γ (U/ml)	Griseofulvin (μg/ml) 0 HLA-DR	0 ICAM-1	1 0 HLA-DR	1 0 ICAM-1	4·0 HLA-DR	4·0 ICAM-1	10 HLA-DR	10 ICAM-1
0	0	0	0	0	0	0	0	0
400	84·9±4·0	71·3±7·6	24·0±4·6*	74 0±6·3	31·2±3·1*	76·0±10·6	10·5+6·5*	72·4±8·4

Keratinocytes were incubated with IFN-γ (400 U/ml) and/or griseofulvin for 72 h. HLA-DR or ICAM-1-positive cells were counted Values are means ± SD.

* $P<0{\cdot}01$ compared with IFN-γ alone

Table 4. Percentage of HLA-DR positive keratinocytes Time of action of griseofulvin on HLA-DR expression

IFN-γ (U/ml)	Griseofulvin (10 μg/ml) 0	0–72 h	24–72 h	48–72 h
0	0	0	0	0
400	70·8±2·0	4·3±1·1*	64 9±8·5	62 9±11·0

Values are means ± SD.

* $P<0{\cdot}001$ compared with IFN-γ alone.

are shown in Table 3. Griseofulvin decreased IFN-γ-induced HLA-DR expression even at a dose of 1·0 μg/ml and HLA-DR expression was strongly inhibited at 10 μg/ml. Griseofulvin did not induce expression of HLA-DQ or -DP (data not shown). Griseofulvin, with or without IFN-γ, did not affect the viability of keratinocytes, which was always >90%.

Effect of griseofulvin on IFN-γ-induced ICAM-1 expression

As shown in Table 3, no alteration of ICAM-1 expression was observed even at a dose of 10 μg/ml of griseofulvin.

Time of action of griseofulvin on HLA-DR expression

Griseofulvin decreased the IFN-γ-induced HLA-DR expression only when it was present at an early stage of incubation (Table 4). When griseofulvin was added after 24 h, it did not affect IFN-γ-induced expression of HLA-DR.

Discussion

Griseofulvin is a derivative of *Penicillium* moulds, and was first isolated from *Penicillium griseofulvum*.[14] Structurally, the compound is 7-chloro-2′ 4, 6-trimethoxy-6′-methylspirobenzofuran-2(3H),1′-(2)cyclohexene-3, 4′-dione. It has been used extensively as an antifungal agent in the treatment of superficial dermatomycoses in humans and animals.[15] It also has other influences on mammalian tissues, including induction of mitotic arrest at metaphase,[16] potentiation of the toxic effects of colchicine[17] and atrophy and inhibition of growth of testicular epithelium.[18] An increased incidence of skin tumours after methylcholanthrene application in mice,[19] thyroid tumours in rats, and hepatomas in mice[20] have also been reported with griseofulvin treatment. Immunological effects of griseofulvin, however, have not been described.

Because of reports of the effectiveness of griseofulvin as a treatment in lichen planus,[8–11] the influence of griseofulvin on MHC class II Ag and ICAM-1 Ag expression of keratinocytes induced by IFN-γ was investigated in this study, with the following results: (i) IFN-γ induced HLA-DR and ICAM-1 expression, but not HLA-DP or -DQ; (ii) griseofulvin alone did not induce HLA-DR, -DP, -DQ or ICAM-1 expression; (iii) griseofulvin decreased the IFN-γ-induced HLA-DR expression, but not ICAM-1 expression. In the time-course experiments, it was found that: (iv) griseofulvin decreased the IFN-γ-induced HLA-DR expression only when it was present at an early stage of incubation and that (v) griseofulvin did not affect the IFN-γ-induced ICAM-1 expression on keratinocytes.

Thus, a differential effect of griseofulvin on IFN-γ-induced HLA-DR and ICAM-1 expression was observed, and these results were very similar to those obtained with 1,25-dihydroxyvitamin D_3, as previously described.[7] This suggests that griseofulvin could be used as an immunomodulator, like 1,25-dihydroxyvitamin D_3,[21] and might be useful in the treatment of some skin diseases such as psoriasis vulgaris. These studies are now in progress.

Acknowledgment

We thank Dr Masutaka Furue for his constructive review of this manuscript and Miss Kyoko Kagami for her expert secretarial assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (K.T.).

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British Journal of Dermatology (1992) 127, 262–265.

Modern treatment of warts: cure rates at 3 and 6 months

J.BERTH-JONES AND P.E.HUTCHINSON
Department of Dermatology, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

Accepted for publication 16 April 1992

Summary

Four-hundred consecutive referrals with viral warts of the hands and/or feet were investigated to determine the cure rate from a combination of cryotherapy, keratolytic wart paint and paring. For treatment failures after 3 months, the value of continuing cryotherapy and of additional treatment with the immunomodulator inosine pranobex were assessed.

Subjects were treated for 3 months with wart paint and cryotherapy and were randomized to receive, or not, paring in addition. Those who did not respond by 3 months were randomized to receive, or not, 3 months further cryotherapy, and to receive inosine pranobex 60 mg/kg/day for 1 week each month, or matching placebo.

Fifty-two per cent of subjects were cured by 3 months. The chance of cure was inversely related both to the length of history and to the diameter of the largest wart. Paring improved the cure rate for plantar warts but not for hand warts. During the second 3 months the cure rate fell to 41%. Neither cryotherapy nor inosine pranobex significantly improved this response.

The treatment of warts is both time consuming and expensive. Although the majority of cases are probably treated in general practice,[1] a recent survey found that warts represented 21% of referrals to a dermatology department, and the treatment and follow-up of these patients constituted 19% of the workload.[2]

Previous studies by Bunney *et al.*[3] extensively investigated the 3-month cure rates from the use of wart paints, cryotherapy at 3-week intervals, and a combination of both treatments. The results of these studies appeared to indicate that treatment was highly beneficial, with cure rates of over 80% for plantar warts treated with wart paint alone, and 78% for hand warts treated with a combination of wart paint and cryotherapy. However, no results have been reported for continued therapy in treatment failures at 3 months.

Inosine pranobex is an immunomodulating agent which has been reported to improve the response of genital warts to conventional treatment.[4,5] There have been no studies published concerning its use for common warts. This drug is expensive and could be considered only for warts unresponsive to conventional treatment.

The aims of this study were to determine our 3-month cure rate and, in treatment failures, to assess the value of continuing cryotherapy and of additional treatment with inosine pranobex. An opportunity was also provided to study predictor variables which might facilitate more efficient targeting of treatment, and to assess the value of paring the surface of warts during the first 3 months.

Methods

Four-hundred consecutive referrals to our department requiring treatment for common warts of the hands and/or feet were enrolled in the study. Subjects of all ages and either sex were included. Plane warts were excluded. Subjects or parents were required to give signed consent. In order to assess possible factors which might influence response, the age and sex of each subject were recorded, together with previous treatment used, the site, size and number of the warts and the length of time they had been present. In view of the large sample size it was not considered necessary to stratify patients into treatment groups using these characteristics.

Subjects were all treated by cryotherapy and wart paint and were randomized to receive, or not, paring of warts of 5 mm or greater diameter. No attempt was made to pare warts smaller than this. Paring was performed, prior to cryotherapy, with a scalpel blade until capillary bleeding was visible within the wart.

Cryotherapy was performed using the technique described by Bunney *et al.*[3] Liquid nitrogen was applied using cotton wool buds until a halo of ice was visible around the circumference of each wart. Patients were treated at intervals of 3 weeks.

Correspondence: Dr J.Berth-Jones.

Wart paint, 16·5% salicylic acid, 16·5% lactic acid in flexible collodion (Salactol®) was applied daily by subjects (or parents), under occlusion when practicable. The importance of regular application and of removing dead skin prior to each application with pumice or emery board was emphasized.

Treatment continued to be performed by all those normally treating warts in our department. Approximately half the treatments were performed by junior medical staff, and half by senior nursing staff.

A frequent problem with studies of wart treatment is the default rate. We attempted to reduce this by stressing the importance of regular attendance to our subjects, and also by providing each of them with a form and reply-paid envelope to send us if they were not returning for any reason, to indicate whether or not they were cured.

At 3 months, those patients who had failed to respond continued to use wart paint and remained under 3-weekly review. All these subjects were randomized to receive, or not to receive, further cryotherapy, and in addition, all were further randomized to receive oral inosine pranobex 5 mg/kg daily or matching placebo for 1 week each month. Paring was continued only for plantar warts over 5 mm in diameter.

At the end of each 3-month treatment period subjects in whom no visible evidence of warts remained on review, or who informed us by post that they had resolved, were classed as 'cured'. Those who completed the protocol and were not cured were classed as 'not cured'. Those who defaulted either by attending late, or by not attending without informing us that they were cured, were classed as 'defaulters'. Subjects were rejected if they attended more than 1 week late on any occasion, or more than 3 days late on two occasions during each 3-month period.

The effects of the age, maximum wart diameter, length of history and number of warts per individual on the 3-month cure rate were tested by the Mann–Whitney *U*-test. The significance of responses to treatment were tested in each case by χ^2 tests. Results at 6 months were tested using a 2 × 4 contingency table with 3 degrees of freedom in a test of overall equality, and in addition, 2 × 2 contingency tables were used to assess the effect of each individual treatment.

Results

Demographic data

Two-hundred and three subjects were male and 197 female. Ages ranged from 5 to 85 years, with most (54%) in the range 11–25. The majority had received previous treatment, usually with keratolytic agents (Table 1). Two-hundred and forty-five subjects had warts on the hands only, 99 on the feet only, and 56 on both sites.

Table 1. Previous treatment

Keratolytics	312
Cryotherapy	24
Paring	10
Cautery	3
Excision	1
None	60

Three-month cure rate

At 3 months, 42% of subjects had been cured, 39% were not cured, and 19% had defaulted. The proportion of patients who defaulted was similar in all demographic and treatment groups. If cure rates are expressed as the proportion of those completing the treatment protocol (Table 2), there was a 52% overall response rate (95% confidence interval (CI) 47–58%).

Neither the sex and age of the subject, nor the number of warts per individual had any apparent influence on the chance of cure. Of those who had received no previous treatment, 58% were cured (non-defaulters $n=50$, 95% CI 44–72%). This was not significantly different from the overall cure rate. The longer the warts had been present the lower was the chance of cure ($P<0{\cdot}002$). This was found both for warts on the hands and on the feet. The maximum wart diameter was also a significant predictor of response ($P<0{\cdot}001$), and this was also true of both hand and plantar warts. There was no significant correlation between the length of time warts had been present and maximum wart diameter, so that these two variables appeared to influence the chance of cure independently.

Table 2. Cure rates at 3 months in non-defaulters ($n=323$)

	Pared*	Not pared*	Overall
Hands (%)	46	50	54
Feet (%)	75	39	60
Both sites (%)	32	32	25
Overall (%)	50	44	52

* Non-defaulters with warts of 5 mm or greater diameter ($n=251$).

Table 3. Randomization for wart duration and size (warts over 5 mm)

		Range	Median
Diameter (mm)			
Hands (n=184)	Pared	2–20	7
	Unpared	1–16	6
Feet (n=76)	Pared	3–24	10
	Unpared	1–25	7
Duration (months)			
Hands (n=184)	Pared	2–244	12
	Unpared	1–192	12
Feet (n=76)	Pared	1–360	12
	Unpared	1–200	12

Paring made no difference to the 3-month cure rate for warts on the hands. The cure rate in the pared group was slightly lower at 46%, than in the non-pared group (50%) (non-defaulters, $n=147$, $P=0{\cdot}75$) for warts of 5 mm or greater diameter. However, paring did make a significant difference to plantar warts, improving the success rate from 39 to 75% ($n=60$, $P<0{\cdot}025$), for warts of the same size. This was independent of the predictive effect of duration or wart diameter, as subjects were effectively randomized for these variables (Table 3).

Six-month cure rate

At the end of 3 months 155 treatment failures entered the next phase of the study. Eighty-nine of these subjects had warts on the hands only, 32 on feet only, and 34 on both sites. Overall, 31% were cured, 44·5% were not cured and 24·5% defaulted. Of those who did not default during this period, the overall cure rate was 41% (95% CI 36–46%), 43% on the hands, 48% on the feet, 24% for subjects with warts on both sites. Cure rates for each treatment group are shown in Table 4. There was no significant benefit from cryotherapy or inosine pranobex.

Table 4. Cure rates at 6 months in non-defaulters ($n=117$)

Treatment group	Inosine pranobex	Placebo	Overall
Cryotherapy (%)	52	38	43
No cryotherapy (%)	32	44	38
Overall (%)	43	40	41

Discussion

The 3-month cure rate was low in this study when compared with previously reported results.[3] The lack of paring in 50% of individuals may be a partial explanation, but even allowing for this, the overall cure rate was still lower. Possible explanations include unrecognized differences in the treatment between the two studies, differences in the predominant wart virus strains, differences in the patient population, or a combination of these. The treatment was administered in our clinics by staff with varying experience in dermatology, so that results would be representative of routine practice. In previous studies treatment has been performed exclusively by those with a special interest and enthusiasm. However the treatment was carefully standardized, and in particular we closely followed the cryotherapy technique used by Bunney. It would therefore appear unlikely that differences in treatment technique are responsible for the relatively low cure rate. There is some evidence that geographical variations may occur in predominant wart virus strains,[6] and it is possible that there are differences in response to treatment between different strains of human papillomavirus (HPV). Plane warts (associated with HPV type 3) are widely regarded as particularly resistant to treatment, but no warts with this morphology were included in the current study. Mosaic plantar warts may also respond differently, but there were only six of these in this study and there was no marked difference in response rate between these and other plantar warts.

The comparatively low 3-month cure rate appears most likely to be a result of differences in the patient population. The vast majority of our patients had received previous treatment. It appears likely that we were treating a population partially selected by failure to respond to previous treatment prior to being referred to us. This hypothesis is supported by our finding that the chances of cure were inversely related to the length of time the warts had been present. This was also noted by Bunney, and the effect was highly statistically significant in the current study ($P<0{\cdot}002$). An increase in the proportion of resistant warts being referred has also been reported by Bunney.[3] However, the response rate among patients who had not used any previous treatment was not significantly higher, suggesting that this may not be the entire explanation.

Another variable with a statistically significant effect on the 3-month cure rate was the diameter of the largest wart. This has not previously been investigated. The larger the maximum wart diameter, the less likely was the patient to be cured. This was independent of the length of history because there was no correlation between size and duration. Our results did not confirm previous observations that the chance of cure was related to the number of warts present and to the age of the patient.[3]

Paring was shown to be valuable as an adjunct to cryotherapy for plantar warts, but was of no value on the hands. This difference between hands and feet is underlined by the fact that we were able to demonstrate a significant response to paring in a relatively small sample of plantar warts, but not in a considerably larger sample of patients with hand warts. Plantar warts tend to produce far more callosity than hand warts. This finding is therefore consistent with the observation that keratin is an effective insulator and might be expected to reduce the efficacy of cryotherapy.[7] The result is not surprising, but has not been formally demonstrated before. It is important to know that the considerable time taken to pare warts on the feet is justified.

The overall response rate fell from 52% during the first 3 months of the study, to 41% during the second 3-month period. There was therefore a diminishing return as treatment continued. Most importantly, cryotherapy did not significantly improve the cure rate obtained from the use of wart paint alone after 3 months. Inosine pranobex is a costly treatment, and the results of this study did not show that it produced any improvement in the cure rate. Unless the benefit was considerable neither continuing cryotherapy nor the use of inosine pranobex would seem cost effective. Although it remains possible that these treatments produce a small effect, it is unlikely that a useful improvement in response rate would not have been detected by this study. We would suggest that a useful response would be reflected by 20% more subjects being cured by an additional treatment. We estimate that the power of the second phase of this study, in which 117 subjects completed the protocol, was more than 85% to detect a difference in cure rate of 20%.

In conclusion, we have re-examined the currently popular regime for the treatment of warts using a combination of a keratolytic wart paint applied daily and cryotherapy at 3-week intervals and found that we cured 52% of patients by 3 months. Plantar warts, but not hand warts, responded better if pared before freezing. Patients with large warts or warts of long duration were less likely to respond. After 3 months of treatment there was no evidence that continuing cryotherapy or adding inosine pranobex improved the response rate from the relatively safe, painless and convenient use of wart paint alone. This would therefore seem a reasonable time at which to decide either to discharge patients from dermatological care, (to continue, if they wish, using self-treatment with a wart paint), or to change management to treatment with a higher morbidity. Options which are available include the use of repeated freeze–thaw cycles, or more frequent and more prolonged cryotherapy, although the value of these remains to be established in controlled trials. Other treatments which might also be considered include topical 5-fluorouracil[8] and intralesional bleomycin.[9]

Acknowledgments

We thank Dr J.Thompson of the Department of Ophthalmology, University of Leicester for statistical advice. Inosine pranobex and matching placebo were kindly provided by Leo Laboratories Ltd.

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British Journal of Dermatology (1992) 127, 266–271.

A multicentre, parallel-group comparison of calcipotriol ointment and short-contact dithranol therapy in chronic plaque psoriasis

J.BERTH-JONES,* A.C.CHU,† W.A.H.DODD,‡ M.GANPULE,§ W.A.D.GRIFFITHS,¶ R.P.HAYDEY,** M.R.KLABER,†† S.J.MURRAY,‡‡ S.ROGERS§§ AND H.J.JURGENSEN¶¶

**Leicester Royal Infirmary, Leicester, U.K.*
†Hammersmith Hospital, London, U.K.
‡University of British Columbia, Vancouver, Canada
§The Skin Hospital, Salford, U.K.
¶St John's Dermatological Centre, London, U.K.
***University of Manitoba, Winnipeg, Canada*
††Broomfield Hospital, Chelmsford, U.K.
‡‡Victoria General Hospital, Halifax, Nova Scotia, Canada
§§City of Dublin Skin and Cancer Hospital, Dublin, Ireland
¶¶Leo Pharmaceutical Products, Ballerup, Denmark

Accepted for publication 10 April 1992

Summary

Short-contact treatment with dithranol (anthralin) is a widely used treatment for chronic plaque psoriasis. Although effective, it causes staining and irritation, and is therefore inconvenient. Calcipotriol is a recently developed vitamin D analogue which is effective and easy to use. To evaluate the relative efficacy, safety and acceptability of these treatments a multicentre, open, randomized, parallel-group comparison was performed.

Four hundred and seventy-eight patients with chronic plaque psoriasis were randomized to use one of the two treatments for 8 weeks. One group applied calcipotriol ointment (50 μg/g) twice daily. The other used a single application for 30 min each day of Dithrocream® in the highest concentration tolerated. Severity of psoriasis was assessed by modified PASI score at baseline, and after 2, 4, and 8 weeks of treatment. A five-point scale was used by subjects and by investigators as an additional assessment of overall response, and a similar scale was used by subjects to grade acceptability. Total serum calcium was monitored at baseline and after 2 and 8 weeks on treatment.

The mean PASI score fell from 9·1 to 4.7 after 8 weeks on dithranol ($P<0{\cdot}001$), and from 9·4 to 3·4 on calcipotriol ($P<0{\cdot}001$). The difference between the two treatments was significant in favour of calcipotriol at 2 weeks ($P<0{\cdot}001$), and remained so at subsequent assessments. At 8 weeks the difference between mean improvements in scores for the two groups was 1·6 (95% confidence interval 0·5–2·7). Efficacy grading by subjects and investigators, and acceptability grading by subjects, were all significantly better for calcipotriol. The mean serum calcium showed no increase in either group.

Calcipotriol was more effective and better accepted than short-contact dithranol. Both treatments appeared free from serious adverse effects.

Dithranol (anthralin) has been used for treating chronic plaque psoriasis since it replaced the naturally occurring analogue, chrysarobin, in 1916. In a wide variety of formulations and treatment regimes, it has proved to be one of the safest and most effective topical treatments available, and is widely regarded as the first-line treatment of this disease. The principal disadvantages are irritancy and staining. The best results are obtained during in-patient treatment with regimes such as that described in 1953 by Ingram, comprising daily tar baths, followed by ultraviolet irradiation and 24-h treatment with increasing concentrations of dithranol in Lassar's paste.[1]

Correspondence: Dr J.Berth-Jones, Department of Dermatology, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

This regime is impractical for most cases of mild or moderately severe psoriasis, and numerous attempts have been made to use dithranol in more convenient formulations and treatment schedules. Cream formulations have been shown to be effective,[2,3] and are easier to remove than pastes and ointments. Shorter application times have been shown to be almost as effective as all-day treatment[4,5] and can be used on an out-patient basis or applied at home. Short-contact self-treatment with a cream formulation has therefore become a very popular first-line treatment.

Calcipotriol is a vitamin D analogue which has already been extensively investigated in the topical treatment of psoriasis. The efficacy and safety of this compound have been demonstrated in double-blind, placebo-controlled studies.[6,7,8]

In two large double-blind trials calcipotriol ointment in a concentration of 50 μg/g has been compared with 0·1% betamethasone 17-valerate ointment for the treatment of chronic plaque psoriasis. In the first of these, a right/left comparison, calcipotriol produced a significantly greater reduction in the PASI score.[9] In the second, a parallel group study, no significant difference in reduction of PASI score was detected between the two treatments although patients graded calcipotriol significantly more effective.[10]

The purpose of this study was to compare the relative efficacy, safety and acceptability of short-contact dithranol used at optimal concentration in a popular formulation, with calcipotriol ointment (50 μg/g) applied twice daily, in the treatment of chronic plaque psoriasis.

Methods

Study design

The trial was a multicentre, randomized, open, prospective, parallel-group comparison of two treatments, and was performed in the United Kingdom, Canada and Ireland.

Subjects

Out-patients, of either sex and aged 18 or over, attending dermatology clinics for treatment of chronic stable plaque psoriasis, were recruited. All gave informed consent.

Patients who were known not to respond to dithranol or calcipotriol were excluded. Other exclusion criteria were: systemic treatment, including PUVA, during the 2 months preceding the study; hypercalcaemia; abnormal renal or hepatic function; intake of more than 400 units daily of vitamin D, or calcium tablets; sensitivity to any component of Dithrocream® or calcipotriol ointment; concurrent medication likely to affect the outcome of the trial. Females were excluded if they were pregnant or not using adequate contraception.

Visit schedule

Subjects attended a 'screening visit', at which all treatment for psoriasis except an emollient cream was discontinued, and initial investigations were performed. After a 2-week washout period subjects attended a baseline visit. Those who met all entry criteria were randomly allocated to one of two treatment groups. Subjects were then reviewed at 2, 4 and 8 weeks of active treatment.

Randomization

Treatment was randomized in balanced blocks of four using computer-generated random numbers.

Treatment

Calcipotriol ointment (50 μg/g, Dovonex®) was applied twice daily to all lesions below the head and neck, except flexures, and lightly rubbed in. A maximum of 100 g was used each week.

Dithranol cream (Dithrocream®) was applied daily to all lesions below the head and neck, except flexures, rubbed in until completely absorbed and left for 30 min before removal in a shower or bath. Treatment was commenced with the highest concentration which the patient was known to tolerate, or 0·1% in subjects who had not previously used dithranol. The concentration was increased each week by the patient, to 0·25, 0·5, 1 and 2%. Treatment was maintained with the highest concentration tolerated.

Compliance

To optimize compliance, all subjects were given written instructions for their allocated treatment. Records were kept of all treatment dispensed and subjects were questioned about compliance at each visit.

Clinical assessments

At baseline, 2, 4 and 8 weeks the severity of psoriasis was assessed using the psoriasis area and severity index

(PASI) scoring system.[11] The head and neck were not assessed.

At the end of the study, both the subject and investigator graded the overall response on the following scale: worse, no change, slight improvement, marked improvement, completely cleared. Subjects were also asked to grade the cosmetic acceptability of the treatment on the following scale: very poor, poor, acceptable, good, excellent.

Laboratory assessments

Full blood count and serum biochemistry, including total calcium, were checked on screening, and after 2 and 8 weeks on each treatment.

Adverse events

At each visit subjects were questioned about adverse events without using leading questions which would suggest any specific symptomatology. Subjects were also examined at each visit in order to detect any adverse reactions.

Statistics

The sample size was calculated on the basis of the percentage change in PASI score between baseline and the end of the study. It was estimated that the improvement in PASI score on treatment would be about 70%, with a standard deviation of 35%. In order to detect a 10% difference in response between groups with a power of 80% at a significance level of 5% using a two-tailed test it was estimated that 200 subjects would be required in each group.

The significance of mean changes in PASI scores within each group between baseline and each subsequent visit was assessed using the paired *t*-test. Differences between mean scores, and differences in mean response between the two groups at each visit were evaluated using the unpaired *t*-test. The chi square and exact tests were used, as appropriate, to evaluate the proportion of patients in each treatment group who reported each grade of response and cosmetic acceptability, and who had adverse events recorded. Two-tailed tests were employed throughout. All tests were corroborated using non-parametric methods.

Table 1. Comparison of baseline data between treatment groups

	Dithranol group	Calcipotriol group	*P*
n	239	239	
% Male	52	57	0·36
Mean age (SD)	43 (16)	45 (15)	0·54
Range	18–85	18–85	
Duration of psoriasis in years (SD)	18 (12)	17 (12)	0·17
Previous dithranol treatment	176 (74%)	182 (76%)	0·6
Baseline PASI score (SD)	9·1 (6·1)	9·4 (6·5)	0·69

Results

Demographic data

Four-hundred and ninety-four subjects were recruited at 47 centres. Sixteen were excluded after screening investigations, leaving 478 who were randomized. The two treatment groups were of equal size and were well matched in terms of age and sex distribution, duration of disease and disease severity at baseline. The majority of subjects in each group had previously used dithranol (Table 1).

Withdrawals

Of 478 subjects randomized, 58 were withdrawn before the end of 8 weeks' treatment. Reasons for withdrawal are summarized in Table 2. The 'other' group at the bottom of this Table includes one subject who was found to have a splenic cyst, one who was unintentionally changed from one treatment to the other during the study and one who was withdrawn in error. One subject in the dithranol group was withdrawn because of a rise in serum calcium above the normal range. Only two

Table 2. Withdrawals

Reason	Dithranol (*n*=239)	Calcipotriol (*n*=239)	*P*
Complete clearing of psoriasis	2	2	NS
Voluntary	7	4	NS
Deterioration of psoriasis	3	3	NS
Medical deterioration unrelated to study	0	2	NS
Cutaneous adverse effects	12	4	0·04
Exclusion criteria	1	1	NS
Hypercalcaemia	1	0	NS
Non-compliance	11	11	NS
Other	3	0	NS

A total of 58 subjects were withdrawn for 67 reasons.

Table 3. Highest concentrations of dithranol tolerated

Concentration (%)	% Subjects ($n=239$)
Unable to tolerate 0·1	1
0·1	9
0·25	11
0·5	15
1·0	21
2·0	41
Unknown	3

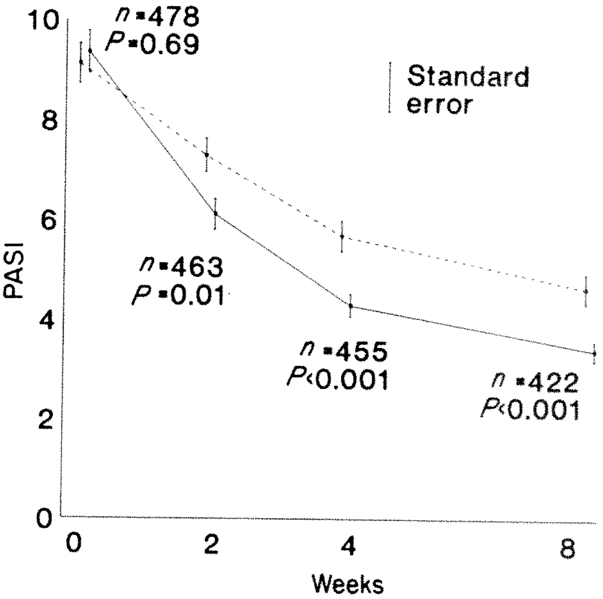

Figure 1. Mean PASI scores. Final data from two subjects withdrawn after week 6 are included at week 8. – – – – –, dithranol; ——— calcipotriol.

subjects were withdrawn after receiving more than 6 weeks of treatment. Data obtained from these subjects at the time of withdrawal were included in the 'week 8' results.

Compliance

The highest strength of dithranol tolerated by subjects is shown in Table 3. Both treatments were generally used as directed. Only 22 subjects were considered not to have complied and these were withdrawn (Table 2). No subject was prescribed more than 90 g of calcipotriol ointment per week.

Response to treatment

During the 8 weeks of the study the mean PASI score fell from 9·1 (standard deviation 6·1, $n=239$) to 4·7 (4·4, $n=208$) in patients on dithranol ($P<0·001$), and from 9·4 (6·5, $n=239$) to 3·4 (2·7, $n=214$) in those on calcipotriol ($P<0·001$). Mean PASI scores at each visit are shown in Figure 1 and, for information, P values for the differences between the treatment groups are shown. However, the principal analysis of the difference between the two groups was based on the difference between mean improvements in PASI score. This was significant in favour of calcipotriol at 2 weeks ($P<0·001$), and remained so at each subsequent assessment. At 8 weeks, this difference was 1·6, 95% confidence interval (CI) 0·5–2·7.

Grading of response by subjects and investigators at the end of treatment is shown in Figure 2. There were again significant differences in favour of calcipotriol in both subjects' and investigators' assessments ($P<0·001$). A grading of 'marked improvement' or 'completely cleared' may be regarded as an unequivocal response to treatment. These grades were selected by 54% of subjects who used dithranol ($n=227$) as against 78% of subjects who used calcipotriol ($n=231$), a difference of 24% (95% CI 16–33%). Investigators assessments were very similar, 'marked improvement' or 'completely cleared' in 51% on dithranol ($n=227$) and 78% on calcipotriol ($n=231$), a difference of 28% (95% CI 19–36%).

There were no significant differences between the participating countries in responses to either treatment.

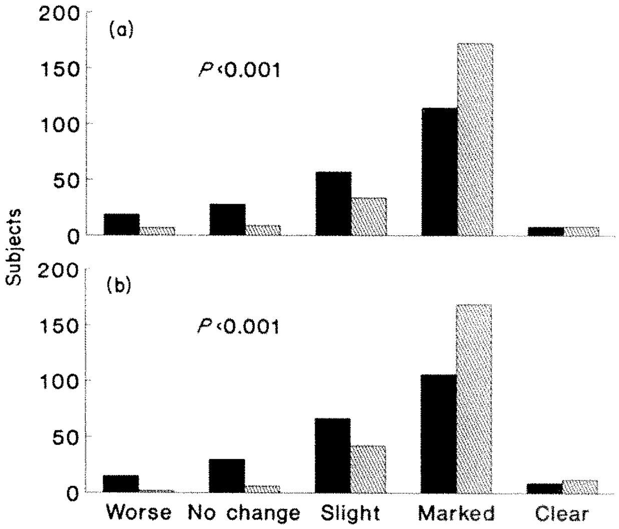

Figure 2. (a) Efficacy grading (subjects). (b) Efficacy grading (physician). ■, dithranol ($n=227$); ▨, calcipotriol ($n=231$).

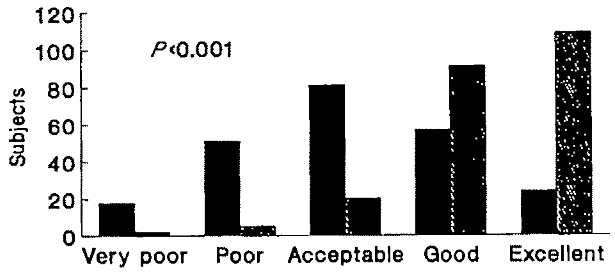

Figure 3. Acceptability grading. ■, dithranol ($n=227$); ■ calcipotriol ($n=231$).

Cosmetic acceptability

Subjects' grading of cosmetic acceptability of the treatments is shown in Figure 3. This was also significantly better for calcipotriol ($P<0{\cdot}001$).

Adverse events

Adverse effects on the skin are summarized in Table 4. Irritant reactions affecting lesional or perilesional skin were common. Reactions on the face were usually erythematous, pruritic, or eczematous in nature. At other remote sites similar reactions were seen, and five subjects on calcipotriol noticed folliculitis or pustules. In addition, the following events, variously classified as probably, possibly or unlikely to be related to treatment, were reported by investigators during the study. One subject in each group developed an episode of renal colic. Single subjects on dithranol complained of coryza and of nausea. Single subjects on calcipotriol complained of headache, of lassitude and of 'stomach flu'.

Laboratory monitoring

In the group using dithranol, the mean serum calcium (with SD and n value) was 2·38 (0·11, 237) at baseline, 2·37 (0·12, 226) at 2 weeks and 2·37 (0·10, 201) at 8 weeks; in the calcipotriol treated group these values were very similar; 2·38 (0·1, 238) 2·37 (0·12, 226) and 2·37 (0·11, 209), respectively. In no subject did haematological or biochemical parameters give rise to concern over adverse effects of treatment. The highest recorded levels of serum calcium were 2·79 on calcipotriol and 2·99 on dithranol. In no subject was there a sustained increase above the normal range. One patient was withdrawn from the dithranol group with a marginal and clinically unimportant rise in serum calcium above the normal range.

Table 4. Cutaneous adverse effects

Symptom	Dithranol (%) ($n=239$)	Calcipotriol (%) ($n=239$)	P
Burning or irritation of lesional or perilesional skin	115 (48)	48 (20)	<0·001
Facial erythema or rash	1 (0·4)	10 (4)	0·006
Other cutaneous symptoms at sites remote from treatment	11 (5)	26 (11)	0·01
Total	127 (53)	84 (35)	<0·001

Discussion

Calcipotriol reduced the severity of psoriasis, as measured by the PASI score, significantly better than short-contact dithranol therapy. Grading of efficacy both by patients and by investigators also yielded highly significant differences in favour of calcipotriol. In addition, the cosmetic acceptability of calcipotriol was graded significantly better than dithranol by the subjects. Calcipotriol also produced fewer irritant reactions in lesional and perilesional skin. Reactions at remote sites such as the face were infrequent, but occurred more often on calcipotriol than on dithranol. These caused few withdrawals and both treatments appeared remarkably free from serious adverse effects.

The main weakness of this study is that it was open and therefore susceptible to observer bias. There may be a tendency in such a study for a new treatment to be received with more enthusiasm than an established treatment. This was, of course, unavoidable. The staining and irritation of normal skin around psoriatic lesions during treatment with dithranol renders it obvious to both patient and observer that dithranol is being used, so that blinding is not possible. The same is also true of all studies in which the efficacy of dithranol is evaluated or compared with other treatments.

The strengths of this study are considerable. The subjects were a well-defined homogeneous group of patients and the sample size was large. Treatments were randomly allocated and the parallel groups were well matched. Treatment was monitored by a cross-section of dermatologists in three countries, all of whom were experienced in the use of dithranol to treat psoriasis. Compliance with both treatments was good, and both were used in a well defined manner. Prospectively defined criteria were used to assess efficacy and safety. The results were unequivocal.

The response to calcipotriol in this study was similar to previous results using the same regime.[9,10] Irritant reactions to calcipotriol in lesional or perilesional skin have also been common in previous studies. The highest incidence so far reported in a short-term study was 19·5%,[10] which is very similar to the level of 20·1% in the current study. However, as in previous studies the majority of these reactions were mild and resolved with continued treatment. Irritant reactions at remote sites such as the face have also been observed in previous studies[7] and are rarely severe. These are presumed to result from inadvertant contamination of these sites with the medication.

Previous studies of the response to short-contact dithranol therapy have generally been numerically much smaller. Results have varied and valid comparisons cannot be made due to numerous differences in important aspects of study design. In-patient treatment cannot be compared to out-patient treatment or to self-treatment at home. The lengths of 'short applications' have varied from 1 min to 2 h, and the overall duration of treatment, when fixed, has ranged from 2 to 12 weeks. Other important differences between trials include the formulations and concentrations of dithranol employed, the types of psoriasis studied, disease severity at baseline and the clinical scoring systems adopted. In addition to these known variables, studies on small numbers of patients are prone to bias in patient selection, as only those subjects known to respond well to dithranol will be recruited. The large sample investigated in the current study is likely to be highly representative of the overall population of patients with chronic plaque psoriasis.

In conclusion, this study has compared two treatment regimes in large and well-matched groups of subjects. Calcipotriol has been shown to be significantly more effective and much better tolerated than a popular regime for short-contact dithranol therapy by self-application at home.

Acknowledgments

This study was sponsored by Leo Pharmaceutical Products, Ballerup, Denmark. Statistical analyses were performed by Mr P.N.Underwood of Leo Laboratories Ltd, Princes Risborough U.K. In addition to the authors, the following investigators participated in the trial. **United Kingdom:** S.J.Adams, R.J.G.Chalmers, M.F.Corbett, I.H.Coulson, W.J.Cunliffe, J.P.Ellis, G.M.Fairiss, D.Goldin, M.Goodfield, C.I.Harrington, C.Holden, P.E.Hutchinson, C.R.Lovell, R.MacKie, D.H.McGibbon, J.A.Miller, M.Price, R.Pye, M.H.A.Rustin, H.K.Shamy, M.J.Tidman, D.M.Tillman, D.I.Vollum, J.Wilkinson, E.Wong, V.M.Yates. **Canada:** J.E.Adam, J.P.Arlette, M.L.Baxter, D.Gratton, R.S.Lester, R.A.Miller, S.Maddin, A.H.Murray, H.E.Murray, C.A.Ramsay, D.Rosenthal, J.B.Ross, G.D.Schachter, R.K.Schachter, N.H.Shear, S.Swiggum, J.W.P.Toole. **Ireland:** L.Barnes, F.Lyons, G.Murphy, S.O.'Loughlin, F.Powell.

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Enhanced association of plasminogen/plasmin with lesional epidermis of bullous pemphigoid

H.M.GISSLER, M.M.SIMON* AND M.D.KRAMER

Institut für Immunologie und Serologie, Immunpathologie, Im Neuenheimer Feld 305, W-6900 Heidelberg, Germany
**Max Planck Institut für Immunbiologie, Stübeweg 51, W-7800 Freiburg, Germany*

Accepted for publication 28 February 1992

Summary

The distribution of plasminogen/plasmin, the central proteolytic component of the plasminogen activator/plasmin system was analysed in lesional skin of bullous pemphigoid by using monoclonal antibodies (MAbs) specific for distinct epitopes of the plasminogen/plasmin molecule. Four groups of MAbs were used: (i) MAbs HD-PG 1 and HD-PG 2, specific for epitopes associated with the lysine-binding sites I (kringle domain 1 + 2 + 3) and II (kringle domain 4) of plasminogen/plasmin, (ii) MAbs HD-PG 6 and HD-PG 7, specific for the lysine binding site I only, (iii) MAbs HD-PG 12 (formerly designated P 2) and HD-PG 18, specific for non-kringle domains of glu- and lys-plasminogen, and (iv) MAb HD-PG 13 which recognizes glu-plasminogen, only. The basal cell layers of normal skin consistently reacted with MAb HD-PG 12, whereas only faint staining was seen with the other MAbs in the same biopsies. In contrast, all anti-plasminogen/plasmin MAbs strongly stained lower and intermediate epidermal cell layers of fully developed bullous pemphigoid lesions.

Plasminogen activators (urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA)) are specific serine proteinases which, by limited proteolysis, convert plasminogen to the active, trypsin-like broad specificity proteinase plasmin. Activity of these proteinases is regulated by inhibitors, i.e. plasminogen activator inhibitors (PAI-1 and PAI-2) as well as plasmin inhibitors (α_2-antiplasmin and α_2-macroglobulin).[1–3] Plasminogen is a 90-kDa single-chain glycoprotein produced in the liver[4] and in the seminiferous tubules of the testes;[5] its normal plasma concentration is 2·2 μmol/l. By immunohistochemistry plasminogen/plasmin has been localized to the basal layer of normal human epidermis;[6,7] there is, however, no evidence for synthesis of plasminogen by keratinocytes *in vivo* or *in vitro*. So-called 'kringle domains'[8] of plasminogen/plasmin mediate its binding to plasma constituents (fibrin,[9] α_2-antiplasmin,[10] tetranectin[11]), structural components of the extracellular matrix[12] as well as cell-surface binding sites.[13,14]

Keratinocytes express plasminogen activators *in vivo*[15–19] and *in vitro*.[20] Via activation of plasminogen, which is abundant in plasma and the interstitial fluid, plasminogen activators are thought to be crucially involved in extracellular proteolysis by keratinocytes. Cell-mediated degradation of extracellular substrates via plasminogen activation is favoured by localization of the involved proteases on the cell surface. When bound to specific cell-surface receptors or binding sites, the interaction of pro-uPA, functionally active uPA, plasminogen and plasmin leads to the generation of cell surface-bound plasmin.[21,22] Cell surface-bound plasmin is efficiently protected against its specific inhibitor α_2-antiplasmin, which is abundant in plasma and the interstitial fluid. Thus, cell surfaces are equipped with the proteolytic potential of plasmin.

Several lines of evidence suggest that plasminogen activation is causally involved in the blister formation of pemphigus vulgaris.[23] Active plasmin leads to acantholysis in skin organ cultures[24] and application of the plasmin inhibitor aprotinin prevents blister formation in an animal model for pemphigus.[25] No such conclusive evidence has been reported for bullous pemphigoid. However, there is circumstantial evidence for involvement of plasminogen activation in this disease. 'Plasmin-like' enzyme activity has been detected in the fluid of bullous pemphigoid skin blisters,[26] and similar to those in pemphigus lesions, keratinocytes in lesional skin of bullous pemphigoid express uPA and tPA.[17] No data is available on the distribution of plasminogen/plasmin in lesional skin of bullous pemphigoid. The present study was undertaken to obtain this data.

Correspondence: Dr M.D.Kramer

Methods

Cell fusion and identification of anti-plasminogen/plasmin MAb-producing hybridoma cell lines

Monoclonal antibodies were prepared as previously described.[27] Their reactivity with distinct forms and molecular domains of plasminogen was determined by ELISA. The following antigen preparations were used: glu-plasminogen (Deutsche KabiVitrum GmbH, München, Germany), lys-plasminogen (Deutsche KabiVitrum GmbH), lysine binding site I (Kringle domains 1+2+3; No. P 1667; Sigma Chemie GmbH, Deisenhofen), and lysine binding site II (Kringle domain 4; No. P 1792; Sigma Chemie GmbH). Flat-bottomed microtitre plates were coated with 100 ng/well of the respective antigen and MAbs bound to this immobilized antigen were then quantified by using peroxidase-labelled anti-mouse immunoglobulin antibody.

Skin biopsies and immunohistochemistry

Immediately after surgical removal, skin biopsies were snap frozen in liquid nitrogen and stored at −70°C until frozen sections (4–5 μm) were cut. Table 1 shows the clinical data of the bullous pemphigoid patients from whom skin biopsies were analysed. Biopsies of normal skin from patients with no signs or history of skin disease served as controls.

The respective MAbs were either used in the form of serum-containing or serum-free culture supernatant (the latter from hybrid cells grown in HL-1 medium [Ventrex Laboratories Inc, Portland, MA, U.S.A.]). Negative control antibodies included antiplasminogen/plasmin MAbs passed over a small column (0·5 ml) containing plasminogen immobilized to sepharose 4B and MAbs recognizing *Borrelia burgdorferi*-associated antigens.[27] Both antibody preparations were negative in immunostaining of skin sections (data not shown).

Table 1. Skin biopsies included in the study. Patients sex, age (in years), titre of anti-basement membrane zone (BMZ) immunoglobulin (Ig) in serum and the intensity of cutaneous Ig deposits at the epidermal BMZ in lesional skin are listed

Biopsy no.	Sex	Age (years)	Anti-BMZ-Ig serum titre	Ig deposits at the BMZ
1	M	56	0	IgG ++
2	M	63	1:640	IgG ++
3	F	?	0	IgG +
4	F	65	1:5	IgG ++/IgA +
5	F	77	0	IgG +
6	F	69	1:2560	IgG ++
7	M	73	1:160	IgG +
8	M	66	1:5	IgG +
9	F	29	0	IgG ++
10	M	81	1:320	IgG ++

Immunohistochemistry was performed by using a commercially available staining kit based on the alkaline phosphatase anti-alkaline phosphatase technique (no. K 670 Kombikit; Dakopatts GmbH, Hamburg, Germany). For counterstaining Mayers haemalaun (No. 9249; Merck AG, Darmstadt, Germany) was used. After mounting of stained sections in Kaisers Glyceringelatine (No. 9242; Merck) photomicrographs were taken using a Zeiss fluorescence microscope with an integrated camera and an Ektachrome 64 (Kodak Ltd, Hemel Hempstead, Herts, U.K.) colour slide film.

Results

Binding characteristics of monoclonal anti-plasminogen/plasmin antibodies

Reactivity of the MAbs HD-PG 1, HD-PG 2, HD-PG 6, HD-PG 7, HD-PG 12, HD-PG 18, and HD-PG 13 was tested on different plasminogen-derived antigen preparations and on control antigens, i.e. tPA and uPA. As summarized in Table 2, four different patterns of reactivity could be distinguished: (i) MAbs HD-PG 1 and HD-PG 2 specific for antigenic epitopes shared by lysine binding sites (LBS) I and II, i.e., kringle domains 1–3 and 4, of plasminogen/plasmin; (ii) MAbs HD-PG 6 and HD-PG 7 specific for LBS II, i.e. an epitope associated with the kringle 4 domain of plasminogen/plasmin; (iii) MAbs HD-PG 12 and HD-PG

Table 2. Reactivity of anti-plasminogen/plasmin MAbs used for the immunohistological studies. Four groups of MAbs (I–IV) were classified according to their reactivity with four different plasminogen-derived antigen preparations: glu-plasminogen (=plasminogen containing a N-terminal glutamic acid residue), lys-plasminogen (plasminogen containing a N-terminal lysine residue), lysine binding site I (LBS I=formed by kringle domains 1–3), LBS II (formed by kringle domain 4). As control antigens uPA and tPA were tested. Reactivity was evaluated by the ELISA technique as described in Methods section. MAb HD-PG 12 is identical to the previously described MAb P2[7]

Group no.	Anti-PG MAb designation	Reactivity with glu-PG	lys-PG	LBS I	LBS II	uPA/tPA
I	HD-PG 1; HD-PG 2	+	+	+	+	−
II	HD-PG 6; HD-PG 7	+	+	+	−	−
III	HD-PG 12; HD-PG 18	+	+	−	−	−
IV	HD-PG 13	+	−	−	−	−

18 specific for epitopes associated with non-kringle domains of glu- and lys-plasminogen; and (iv) MAb HD-PG 13 specific for glu-plasminogen only. The respective anti-plasminogen/plasmin MAbs do not cross-react with either uPA or tPA.

Reactivity of monoclonal anti-plasminogen/plasmin antibodies on frozen skin sections

MAbs HD-PG 2, HD-PG 6, HD-PG 12 (HD-PG 12 was formerly designated P2[7]) and HD-PG 13 that displayed distinct reactivity on purified plasminogen-derived antigen preparations (Table 2) were tested on frozen sections of lesional bullous pemphigoid skin; the clinical data of bullous pemphigoid patients is summarized in Table 1. In uninvolved skin areas most distant from the apparent blister formation, i.e. skin areas with no histologically or immunohistologically detectable pathological alterations, only MAb HD-PD 12 displayed consistent staining of the lower epidermal cell layers (see Table 3 and Fig. 1d). The observed staining pattern is in agreement with immunofluorescence studies previously performed with this MAb.[7] In contrast, in perilesional skin areas (i.e. directly adjacent to apparent blister formation) and in lesional skin areas (i.e. in the area of subepidermal blistering), staining was observed with all four antibodies (summarized in Table 3; exemplified for MAb HD-PG 2 in Fig. 1b and c). This was most pronounced for MAbs HD-PG 2, HD-PG 13, and HD-PG 6 which strongly stained all (10/10) biopsies from blistered skin areas but only weakly stained four out of 10 (HD-PG 2, HD-PG 13) or two out of 10 (HD-PG 6) biopsies from uninvolved skin areas (Table 3). Higher magnification of stained tissue sections revealed apparently intracellular staining in keratinocytes (see closed arrow in Fig. 1c) and in some staining reactions a particularly strong staining of single epidermal cells was observed (see closed arrows in Fig. 1e for MAb HD-PG 13; designated in Table 3 as 's'). Furthermore, below epidermal blister roofs staining of cellular or extracellular structures was observed (MAb HD-PG 2; see open arrow in Fig. 1c).

Discussion

Our findings provide evidence for an increased association of plasminogen/plasmin with keratinocytes at the site of developing or fully developed blisters in bullous pemphigoid. A quantitative difference of plasminogen/plasmin-specific immunoreactivity in pathological versus normal skin was revealed with different anti-plasminogen/plasmin MAbs (clones HD-PG 2, HD-PG 6, and HD-PG 13) which only showed weak staining in 10 out of 30 sections of non-involved skin areas, faint to marked staining in 22 out of 30 sections of perilesional skin and marked reactivity in all sections of lesional, i.e. blistering, skin areas (summarized in Table 3). MAb HD-PG 12, formerly designated P2,[7] also showed moderately positive plasminogen/plasmin-specific staining in the basal layers of normal skin (Fig. 1d). Whereas MAbs HD-PG 2 and HD-PG 6 recognize epitopes associated with lysine-binding sites of plasminogen/plasmin, MAb HD-PG 13 recognizes an epitope specific for glu-plasminogen, and HD-PG 12 recognizes an epitope shared by glu- and lys-plasminogen and not related to the lysine-

Table 3. Reactivity of anti-plasminogen/plasmin MAbs with skin biopsies of BP patients. Reactivity was evaluated in uninvolved, perilesional and lesional skin areas. MAbs HD-PG 2, HD-PG 6, HD-PG 12, and HD-PG 13 were taken for immunohistochemistry using the alkaline phosphatase anti-alkaline phosphatase technique. −, no reactivity (as in Fig. 1a); +, faint or moderate reactivity (as in Fig. 1b); + +, marked reactivity (as in Fig. 1c). Localization of immunoreactivity was further coded as follows: bc, basal cells (as in Fig. 1d); le, lower epidermis (as in Fig. 1b); ie, intermediate epidermis; s, particularly strong staining of single cells (as in Fig. 1e). *PG 12 is identical to previously described MAb P2[7]

Biopsy no.	Uninvolved	Perilesional	Lesional
	HD-PG 2/HD-PG 6/HD-PG 12*/HD-PG 13	HD-PG 2/HD-PG 6/HD-PG 12*/HD-PG 13	HD-PG 2/HD-PG 6/HD-PG 12*/HD-PG 13
1	−/+bc/+le/−	+ +le/+ +le/+ie/+ +le	+ +le/+ +le/+ +ie/+ +le
2	+le/−/+bc/−	+ +le/+bc/+bc/−	+ +le/+ +le/+ +bc/+bc
3	+bc/+ +bc/+le/−	+ +le/+ +le/+ +le/+ +le	+ +le/+ +le/+ +le/+ +le
4	−/−/+bc/−	−/−/+ +bc/+ +bc	+ +bc/+ +s/+ +bc/+ +le
5	−/−/+bc/+le	−/−/+le/+le	+s/+ +bc/+ +le/+ +bc
6	+le/−/+le/−	+le/−/+le/+ +le	+ +ie/+ +le/+ +ie/+ +le
7	+le/−/+le/+le	+ +ie/+le/+ +ie/+bc	+ +ie/+le/+ +ie/+s
8	−/−/+le/+le	+le/+ +bc/+le/+le	+ +bc/+ +bc/+ +le/+ +bc
9	−/−/+bc/−	+le/−/+bc/+s	+ +bc/+ +bc/+ +bc/+bc
10	−/−/+ie/+le	+bc/−/+ie/+ +bc	+ +s/+ +bc/+ +ie/+ +bc

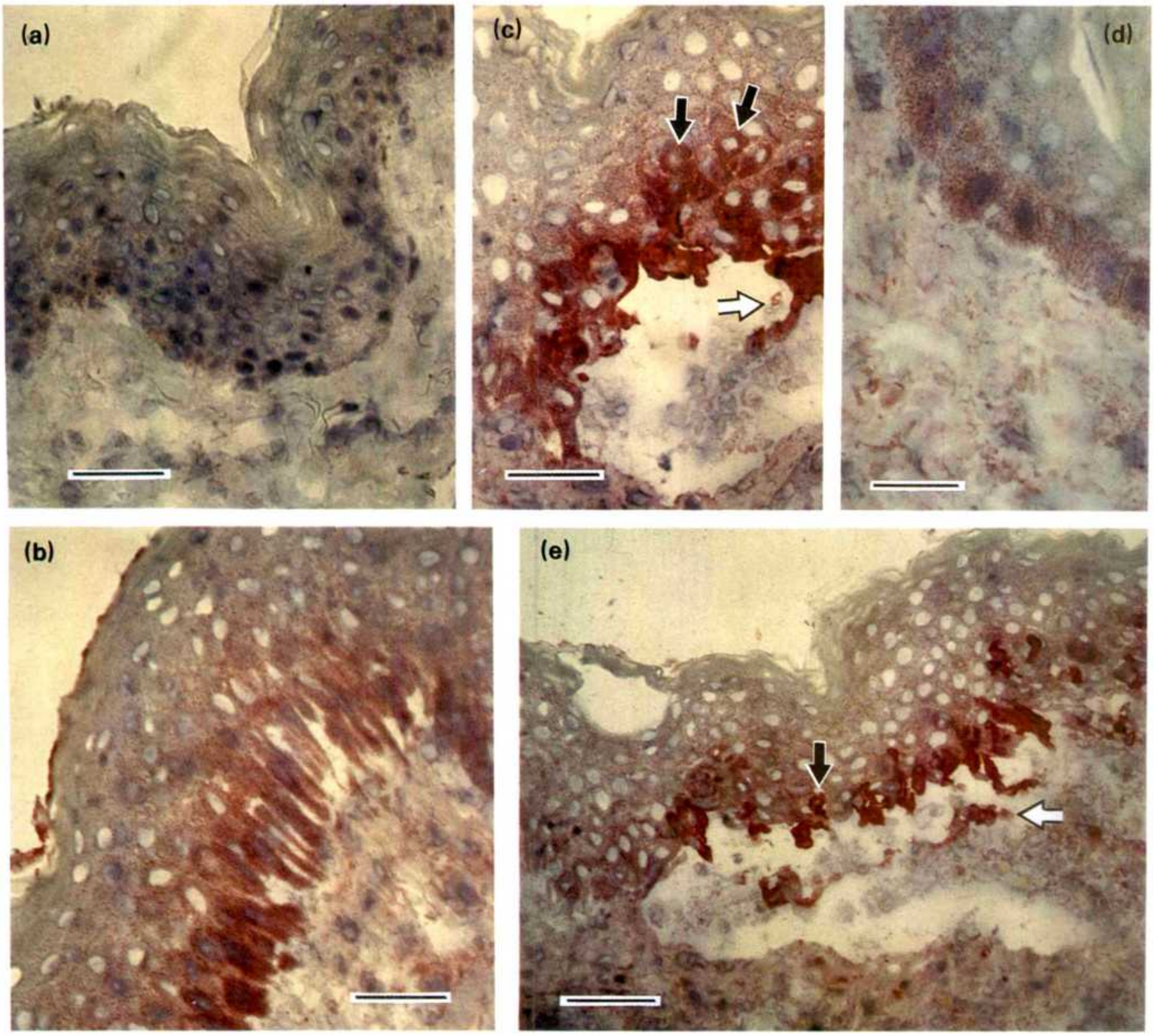

Figure 1. Immunohistological staining of frozen skin sections. Frozen sections (4–5 μm) of bullous pemphigoid skin lesions were stained by using MAb HD-PG 2 (a–c), HD-PG 12 (d), and HD-PG 13 (e) as primary antibody. Bound antibody was visualized by using the alkaline phosphatase antialkaline phosphatase technique. For counterstaining we used Mayers Hämalaun. Scale bars: a, 14 μm; b, 8·5 μm; c, 8·5 μm; d, 7 μm; e, 16·5 μm. (c) Closed arrows indicate positive, apparently intracellular staining reactions for plasminogen/plasmin. (c, e) Open arrows indicate staining of extracellular material. (e) Arrows indicate particularly strong positive staining of single cells (designated s in Table 3).

binding sites (summarized in Table 2). It remains open whether the observed difference in reactivity in immunohistochemistry is somehow linked to the different epitopes recognized by these MAbs. However, the basically similar findings with all MAbs support the conclusion that there is enhanced deposition of plasminogen/plasmin at the site of developing or fully developed skin blisters in bullous pemphigoid.

It cannot be established from the available data whether plasminogen is synthesized by keratinocytes or whether it is taken up from exogenous sources, such as the interstitial fluid. Because there is, at present, no evidence for the synthesis of plasminogen/plasmin by keratinocytes *in vivo* or *in vitro* it could be speculated that plasminogen from plasma or the interstitial fluid is internalized by basal keratinocytes. The putative mechanisms underlying internalization are not known. However, internalization could be mediated via cell-surface binding sites for plasminogen/plasmin, which are known to be expressed on a variety of cell types,[22] but which remain to be identified on keratinocytes. In addition to plasminogen/plasmin receptors, pro-uPA/uPA receptors have been identified on many cell types, including keratinocytes.[28,29] Concomitant binding of pro-uPA/uPA and plasminogen/plasmin to cell surfaces[22] accelerates and focuses the cascade of plasminogen activation.[21] Plasmin can convert pro-uPA to active uPA while bound to the uPA receptor and this receptor-bound uPA can in turn activate surface-bound plasminogen. In contrast to plasmin in solution, surface-bound plasmin is protected against inactivation by plasma-derived inhibitors, such as α_2-anti-plasmin in particular.[14,21] Thus the cell surface is thought to be a preferential site for appearance of plasmin activity.[14]

The data presented here neither provide information of the functional activity of plasminogen/plasmin deposits nor on the disease specificity of this phenomenon. Thus, plasminogen/plasmin deposition might be a secondary phenomenon, without any specific pathogenic significance for bullous pemphigoid skin lesions. However, in view of the strong evidence for a pathogenic role of plasmin in pemphigus it is tempting to speculate that plasmin might also be involved in the mechanisms leading to blister formation in bullous pemphigoid, and that increased localization of plasminogen/plasmin at the site of the dermo-epidermal split is one aspect of these mechanisms. Speculation in this direction is encouraged by the observations that keratinocytes of lesional skin in bullous pemphigoid express plasminogen activators[17] and that the fluid contained in bullous pemphigoid blisters contains plasmin-like proteinase activity.[26] In future studies we will explore the disease specificity of plasminogen/plasmin deposition and the functional state of these deposits in lesional epidermis of bullous pemphigoid.

Acknowledgments

The authors are indebted to Ms U.Schirmer, Ms S. Wentrup, Ms I.Neumann and Ms A.Heidtmann for expert technical assistance and Dr K.Rother for helpful criticism of the manuscript. This work was supported in part by the Deutsche Forschungsgemeinschaft (DFG Kr 931/2-2; Si 214/5-6) and the Bundesministerium für Forschung und Technologie (FKZ 01KG8808/4).

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British Journal of Dermatology (1992) **127**, 278–280.

Dyskeratosis congenita: delay in diagnosis and successful treatment of pancytopenia by bone marrow transplantation

R.J.PHILLIPS, MARY JUDGE, D.WEBB* AND J.I.HARPER

*Departments of Dermatology and *Haematology, Hospitals for Sick Children, Great Ormond Street, London WC1N 3JH, U.K.*

Accepted for publication 15 January 1992

Summary Dyskeratosis congenita is an inherited disorder characterized by nail dystrophy, skin pigmentary changes, mucosal leukoplakia, pancytopenia and an increased incidence of malignancy. Because of a widely held view that the outcome of bone marrow transplantation in dyskeratosis congenita is poor, this treatment option is sometimes not considered when pancytopenia develops. We present a child currently doing well 3 years after bone marrow transplantation, and review the literature.

Dyskeratosis congenita was first described by Zinsser in 1906[1] and was formerly referred to as the Zinsser–Engmann–Cole syndrome. It is an inherited disorder characterized by: nail dystrophy; reticulate pigmentation which typically begins on the neck or trunk; leukoplakia especially of the oral mucosa but also of the mucosal surfaces of the urethra, vagina and ano-rectal junction; pancytopenia and an increased incidence of malignancy.[2,3] Pancytopenia usually develops in the second or third decade and malignancy, particularly oropharyngeal and skin, in the third or fourth decade. Lacrimal duct stenosis and palmoplantar hyperkeratosis are commonly seen, and many other associated features such as telangiectases, hyperhidrosis, sparse hair, poor growth, mild mental retardation and oesophageal anomalies have been described. It is usually inherited in an X-linked manner but families showing autosomal dominant inheritance have been described.[4] Over 100 cases have now been reported.[2]

Of the reported deaths in this condition, 70% have been due to pancytopenia.[2] We report the case of a 5-year-old boy who had been successfully treated with bone marrow transplantation for aplastic anaemia and who was subsequently recognized as having dyskeratosis congenita.

Case report

This boy, born to unrelated Caucasian parents, had mild intrauterine growth retardation, and a birth weight of 2·1 kg. His immediate postnatal course was uneventful and there was no significant family history of skin, nail or haematological problems. By 10 months of age his nails had become abnormal and he was noted to have a hearing impairment. At 2 years of age, he presented with an intraocular bleed, pallor and easy bruising.

Investigations at that time showed haemoglobin 8·6 g/dl, white cell count $5{\cdot}2 \times 10^9$/l with absent neutrophils, and platelet count 8×10^9/l. Bone marrow aspirate and trephine showed aplastic anaemia with normal cytogenetics and no increase in chromosomal fragility. Skeletal survey and renal ultrasound were normal. He was given pre-transplant conditioning with cyclophosphamide (50 mg/kg on each of 4 days) and treated with a matched sibling bone marrow transplant. He had no significant episodes of post-transplant sepsis or graft-versus-host disease, and satisfactory marrow engraftment with donor cells was confirmed by mini-satellite probes. After discharge, he was maintained on cyclosporin prophylaxis against graft-versus-host disease for 18 months. His hair regrew normally 2 months after the transplant, but he subsequently redeveloped diffuse, non-scarring alopecia 2 months later. He has also had problems related to bilateral lacrimal duct blockage, but has otherwise remained well for the ensuing 3 years. The underlying diagnosis of dyskeratosis congenita was not recognized until 18 months after his bone marrow transplant.

Currently he is an active cheerful boy. His nails are all hypoplastic and irregular (Fig. 1) and he has reticulate hyperpigmentation on his neck. He requires a hearing aid and has poor vision in his right eye. His tongue is smooth, but his oral mucosa and teeth are otherwise normal. His height and weight are between the third and tenth centiles, intellectual development is normal and

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Correspondence: Dr J.Harper, Department of Dermatology, Hospitals for Sick Children, Great Ormond Street, London WC1N 3JH, U.K.

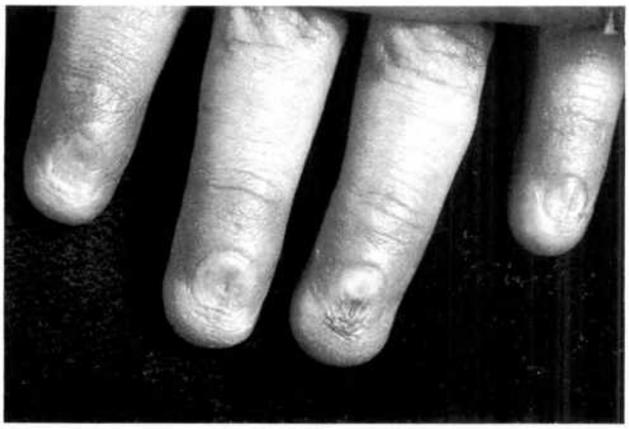

Figure 1. Nails of patient at the age of 4.

his hair has recently regrown. Haemoglobin, white cell count and platelet count all returned to normal after his transplant.

Discussion

There is no pathognomonic test for dyskeratosis congenita, but this boy shows the major features of nail dystrophy, typical pigmentary changes and pancytopenia. He also demonstrates lacrimal duct stenosis (seen in 80%), hyperkeratotic palms, and borderline growth. Alopecia has been described in dyskeratosis congenita[4,5] but in this boy is presumed to have been secondary to his bone marrow transplant. Neither leukoplakia nor malignancy would be expected to be seen at his age. In the absence of a positive family history, he presumably represents a new mutation of either X-linked or autosomal dominant disease.

Both graft-versus-host disease and Fanconi's anaemia were considered as possible diagnoses. Graft-versus-host disease can cause nail changes, alopecia and reticulate pigmentation but there is no supporting evidence for this diagnosis. Moreover, although the age of onset of the skin changes is not known, the nail changes preceded the bone marrow transplant. Fanconi's anaemia is associated with poor growth, pigmentary changes and early onset pancytopenia. However, increased chromosomal fragility is seen in virtually all patients and skeletal abnormalities are seen in most.[3,6] Moreover, nail dystrophy is not a feature of this disease and the pigmentary change is usually diffuse.

There have been eight publications describing patients with dyskeratosis congenita who have been treated with bone marrow transplantation[6–13] but five of these publications appear to refer to the same two boys.[6–10] Our patient is therefore the seventh and youngest to be reported, and details of all seven are given in Table 1.

Patients 1 and 2 both had successful bone marrow transplants but died years later.[6–10] Death was due to late onset thrombotic microangiopathy thought to be secondary to the radiation therapy both had received. Although delayed microangiopathy after bone marrow transplantation has recently been described in many children with leukaemia,[14,15] it has been suggested that patients with dyskeratosis congenita may be at increased risk of this complication and that radiation therapy should be avoided in this group of patients.[10] Patient 3 received a normal conditioning regime and died of overwhelming acute graft-versus-host disease within 2 months. However, he had increased chromosomal fragility, a very unusual finding in dyskeratosis congenita.[11] By analogy with subsequent advances in treatment of Fanconi's anaemia,[6] such patients would now receive alternative conditioning prior to bone marrow transplantation. Patient 4 received busulphan in addition to high-dose cyclophosphamide and died of acute graft-versus-host disease.[12]

No details of the transplantation regime used for

Table 1. Outcome in reported cases of bone marrow transplantation in patients with dyskeratosis congenita

Patient no.	Age/sex (years)	Chromosomal fragility	Donor*	Conditioning†	Outcome
1[6–10]	6/M	Normal	Father	Cy 120, 7 Gy	Died after 7 years
2[6–10]	11/M	Normal	Sister	Cy 150, 6 Gy	Died after 2 years
3[11]	33/M	Increased	Sister	Cy 200	Died after 2 months
4[12]	4/F	Normal	Sister	Cy 240, busul	Died after 3 months
5[13]	11/F	Not given	Sister	Not given	Well after 4 years
6[10]	Child	Not given	Not given	Cy 200	Well after 8 months
7	2/M	Normal	Sister	Cy 200	Well after 3 years

* All HLA-DR matched.

† Cy, cyclophosphamide mg/kg; Gy, irradiation in Grays; busul, busulphan.

Patient 5 are given[13] but this girl was well 4 years after her transplant. Interestingly, as in our case, the diagnosis of dyskeratosis congenita was not made until some years after bone marrow transplantation. Her nail and skin changes had been attributed to chronic graft-versus-host disease, but these changes preceded her bone marrow transplant.[13] Patient 6 was a child who received the same conditioning as our patient and was well 8 months later, but no further details were given.[10]

Although four of the patients in Table 1 have died, dyskeratosis congenita should not be seen as a contraindication to bone marrow transplantation. If Patient 3 with known increased chromosomal fragility is excluded, five of the remaining six patients had apparently successful transplants, and the two patients (6 and 7) who received conditioning with cyclophosphamide alone are well. If late-onset problems as seen in Patients 1 and 2 are prevented by the avoidance of irradiation, bone marrow transplantation may greatly increase life expectancy. Pancytopenia is certainly the major problem in this disease in the first 3 decades of life, with 50% of reported deaths related to infection and 20% related to bleeding problems.[2]

Malignancy is second to marrow failure as a cause of death in patients with dyskeratosis congenita. About 12% of patients had a known tumour at the time of reporting[2] and, as most case reports of dyskeratosis congenita are of patients under 30 years of age, many more are likely to develop a malignancy. Squamous carcinomas of skin or mucosa, Hodgkin's lymphoma and oesophageal and pancreatic cancers have all been described.[2] It has been suggested that etretinate might reduce the incidence of leukoplakia and malignancy in dyskeratosis congenita.[2] Patients with dyskeratosis congenita who are treated with bone marrow transplantation may show a higher incidence of tumours because they avoid early death from marrow failure, but it is also possible that bone marrow transplantation may reduce the inherent risk of malignancy in this disease.

Acknowledgments

We are grateful to Professor J.Chessells and Dr F.Katz who undertook DNA analyses, and to the Department of Medical Illustration for their assistance.

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British Journal of Dermatology (1992) 127, 281–285.

The skin in severe combined immunodeficiency: a case with transient cutaneous presence of γ/δ (TRC1$^+$) T cells

J.H.SILLEVIS SMITT,[*] R.S.WEENING,[†] S.R.KRIEG[‡] AND J.D.BOS[*]

Departments of [*]Dermatology, [†]Paediatrics, and [‡]Pathology, University of Amsterdam, Academisch Medisch Centrum, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

Accepted for publication 10 March 1992

Summary We report the case of a boy with severe combined immunodeficiency (SCID) and serious skin problems. The level of purine 5′-nucleotidase was greatly reduced in the lymphocytes of this patient. To our knowledge, no patients with SCID and this enzyme deficiency have been described previously. The relationship between reduced levels of this enzyme and the immunodeficiency is unclear. This case is also unusual because of the presence of large numbers of T lymphocytes expressing TCR1 (γ/δ) in the skin. Moreover, the presence of so many TCR1-positive cells was not consistent with the low numbers of these cells in the peripheral blood. These cells were not present in skin biopsies taken at a later stage during the course of the disease. An oligoclonal lymphocytosis developed during follow-up, and a monoclonal antibody reactive with these clones was found, indicating that these lymphocytes were present in the skin.

This case report illustrates the benefit of the use of monoclonal antibodies in identifying the cells involved in the cutaneous inflammation in SCID, in order to gain a better insight into the characteristics of these cells.

In severe combined immunodeficiency (SCID), humoral as well as cellular immunity is impaired.[1] The degree of immunodeficiency varies from mild abnormality to complete absence of B- and T-cell function. As a result, there is considerable variation in the age of onset, severity of symptoms and eventual outcome.

These abnormalities have an impact on the skin, particularly on the skin immune system.[2] Most patients tend to have recurrent infections, including skin infections, and fail to thrive. Eczematous reactions are often present,[3] and frequently referred to as 'seborrhoeic dermatitis'. Erythroderma has also been described as a manifestation of combined immunodeficiency.[4] We report a boy with SCID and eczema, which evolved into erythroderma. Initially, the majority of his cutaneous T cells were of γ/δ (TCR1) origin, but in a later phase of the disease these cells disappeared from the skin lesions.

Case report

A diagnosis of SCID was made in a boy, aged 18 weeks, on the basis of clinical and immunological characteristics. The clinical signs were disseminated eczema with superimposed candidiasis in the first weeks of life, bilateral purulent conjunctivitis, periocular abscesses whose contents grew *S. aureus* and *P. mirabilis* on culture, hepatosplenomegaly, and diffuse interstitial pneumonia caused by *Pneumocystis carinii* and cytomegalovirus, and diagnosed by an open lung biopsy.

On admission to hospital the skin eruption consisted of scaling erythematous areas, with poorly defined margins, around the ears and on the chest. Immunological abnormalities included: a sparsity of lymphoid cells and absence of Hassall bodies in a biopsy of the thymus; leucocytosis ($17{\cdot}9 \times 10^9$/l), with 46% polymorphs, 43% lymphocytes, 5% monocytes, 2% eosinophils and 4% atypical lymphocytes; a normal number of T lymphocytes with a CD4/CD8 ratio of 0·10; T-cell antigen receptor (TCR) in 98% of the α/β (TCR2) and in 1% of the γ/δ type (TCR1); no circulating cells bearing the mother's HLA phenotype were found; no detectable

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Correspondence: Dr J.H.Sillevis Smitt, Department of Dermatology, Academisch Medisch Centrum, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

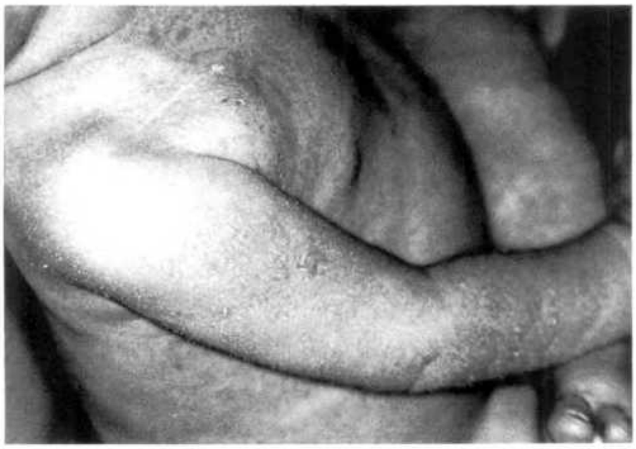

Figure 1. Extensive erythroderma with slight scaling at the age of 16 months.

immunoglobulins; minimal proliferative response of peripheral blood lymphocytes, and normal adenosine deaminase and purine nucleoside phosphorylase activities. Total and ecto-purine 5′ nucleotidase activity were extremely low (total purine 5′ nucleotidase 0·13–0·17 μmol uric acid formation/10^{10} cells/min; controls: 0.75–2.20 μmol uric acid formation/10^{10} cells/min).[5] At the age of 8 months a haploidentical bone-marrow transplantation with T-cell-depleted bone marrow from his father was performed. The marrow did not take; another transplantation was not performed because of severe veno-occlusive disease of the liver.

During follow-up, an increasing peripheral blood lymphocytosis was detected, consisting of 4H3-positive oligoclonal $CD8^+$ T cells. The percentage of $4H3^+$ cells within the T-cell fraction rose from 50 to 85%. 4H3 is a clonotypic MAb that recognizes the TCR of the influenza-specific human T-cell clone 6H5 (KC Kuijpers *et al.* unpublished data).

Eventually he developed an erythroderma of varying severity (Fig. 1). The scalp hair and the eyebrows were lustreless and rather sparse. Topical treatment with corticosteroids or tar ointment produced only slight improvement of the skin. At the age of 2½ years the patient died at home.

When he was first seen, a biopsy (biopsy 1) was taken from a slightly erythematous scaling lesion with an ill-defined border. At 16 months (peripheral blood CD4/CD8 ratio 0·02) and at 26 months (peripheral blood CD4/CD8 ratio 0·1) of age further biopsies (biopsies 2 and 3) were taken from the erythrodermic skin. All biopsies were subjected to histopathology and immunopathology.

Histopathology

All three biopsies yielded a more or less comparable picture: a parakeratotic, slightly acanthotic epidermis with considerable exocytosis. No fungi or yeasts were detected with either PAS or PAS-d staining. The dermis was diffusely infiltrated with mononuclear cells and some eosinophils. The subcutaneous fat was not involved.

Immunopathology

A two-stage immunoperoxidase technique, with 3-amino-9-ethyl-carbazol (AEC) as staining reagent, was employed as the principal immunophenotyping procedure. The processing of tissue specimens, identification of cell types, immunoperoxidase technique and controls (neonatal thymus, lymph node, spleen) used were essentially the same as described previously.[6] Briefly, the fresh-frozen (−70°C) skin specimen obtained by biopsy was sectioned (6 μm), fixed in fresh, pure acetone, and incubated with the monoclonal antibody (MAb) for 60 min, in a humidified atmosphere. Sections were then incubated in a 1:20 dilution of peroxidase-conjugated rabbit anti-mouse Ig for 30 min, in a humidified atmosphere. Staining was achieved by incubation of the sections in freshly prepared acetate-buffer solution (pH 4·9) containing 50 mg AEC in 100 ml buffer, 5 ml dimethylformamide and 0·011% hydrogen peroxide. The staining reaction was visually controlled, and stopped by washing in acetate buffer. Slides were fixed in formalin (4%) and counterstained with Mayer's haematoxylin for 1 min, to give a slight nuclear visualization. Finally, sections were mounted with aquamount.

For the MAbs 11F2, TCR-δ-1, δTCS-1, βF1, 4H3, Tiγ-a and BB3 another immunohistochemical technique was used for immunophenotyping.[7] We used PBs and Leu 4 as controls, in order to see whether the results of the two methods were comparable. Briefly, the fresh-frozen (−70°C) skin specimen was sectioned (6 μm), fixed in fresh, pure acetone at 4°C on a polylysine-pretreated slide, pre-incubated in normal goat serum (1:10 dilution, 10 min) and incubated successively with the MAb for 60 min, rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) (1:25 dilution, 30 min), alkaline phosphatase (AP) anti-AP complex (APAAP; Dakopatts) (1:50 dilution, 30 min), rabbit anti-mouse Ig (20 min) and APAAP (20 min). Between incubations, sections were rinsed in phosphate-buffered saline (PBS). AP activity was detected histochemically: a carmine red colour was developed using naphtol-AS-MX-phosphate (Sigma

Table 1. Monoclonal antibodies used

MAb	Designation	Specificity
Leu 4*	CD3	Pan-T lymphocytes
Leu 2a*	CD8	Suppressor/cytotoxic lymphocytes
Leu 3a+b*	CD4	Helper/inducer lymphocytes
11F2†	TCR1	γ/δ T lymphocytes[8]
TCRδ-1‡	TCR1	γ/δ T lymphocytes[9]
δTCS§	TCR1	γ/δ T lymphocytes, V δ1-J δ1 combination[10]
TiγA¶	TCR1	γ/δ T lymphocytes, V-γ-9[11]
BB3**	TCR1	γ/δ T lymphocytes, V-δ-2[12]
βF1§	TCR2	α/β T lymphocyes[13]
4H3††	TCR of clone 6H5	α/β T lymphocytes
OKT6‡‡	CD1a	Langerhans cells
HLA-DR*	MHC-II	

* Becton Dickinson (Sunnyvale, CA, U.S.A.).

† Gift from J.Borst (Amsterdam, The Netherlands).

‡ Gift from M.B.Brenner (Boston, MA, U.S.A.).

§ T cell Sciences (Cambridge, MA, U.S.A.).

¶ Gift from T.Hercend (Villejuif, France).

** Gift from L.Moretta (Genova, Italy).

†† Gift from K.C.Kuijpers (Amsterdam, The Netherlands).

‡‡ Ortho-Pharmaceuticals (Rariton, New Jersey, U.S.A.).

Chemical Co., St Louis, MO, U.S.A.) as substrate and Fast Red Violet LB (Sigma) as azo dye. Endogenous AP activity was inhibited with 1 mM levamizole (Sigma) added to the incubation medium. Nuclei were counterstained with Mayer's haematoxylin. MAbs employed in this study, as well as their designation and origin, are described in Table 1.[8-13] Epidermal cells were quantified as the number of positive cells present in relation to epidermal length. The percentages of positive cells in the dermal infiltrates were estimated by counting 100 counterstained nuclei in relation to the number of cells surrounded by plasma membrane stained with the MAb.

Table 2. Results of immunophenotyping the subsets of γ/δ cells in the infiltrate of the first biopsy

MAb	Epidermis*	Dermis† (%)
11F2 (TCR1)	0–11	17
TCRδ-1 (TCR1)	34–50	43
δTCS1 (V δ1–J δ1)	3–5	52
Ti-γ-A (Vγ9)	1–6	25
BB3 (Vδ2)	0	3

* Number of positive staining cells per 0·3 mm.

† Number of positive staining cells counting 100 nuclei.

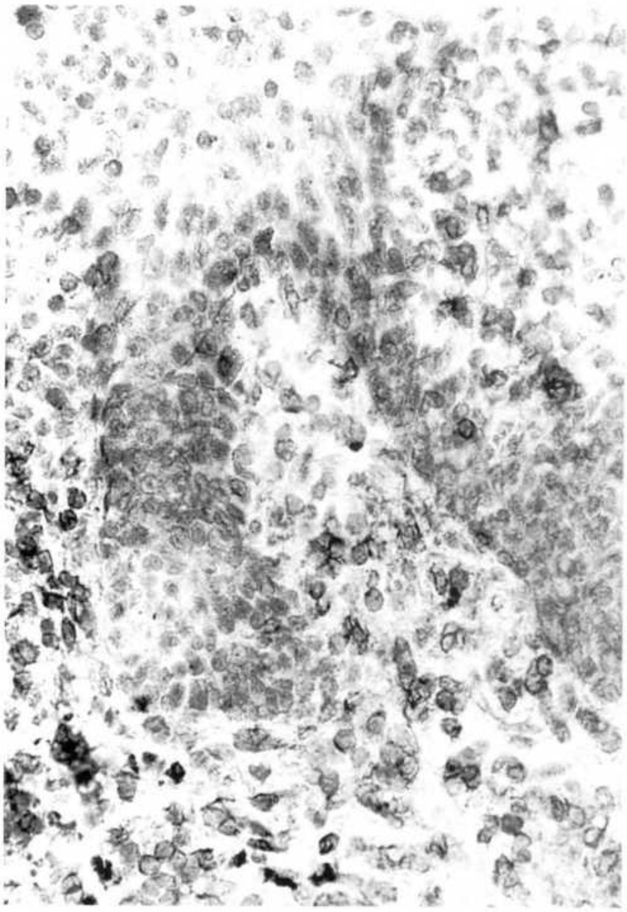

Figure 2. γ/δ T cells in the epidermis and dermis of the first biopsy, stained with the antibody δTCS1 (× 350).

Immunophenotyping the inflammatory infiltrate

Biopsy 1. In this biopsy a considerable number of intraepidermal T cells were CD3$^+$, CD4$^-$ and CD8$^-$, and expressed the TCR1 (γ/δ) receptor. A wide range was found in the number of positively staining cells in different parts of the epidermis. Similar cells were also observed in the dermal infiltrate, particularly directly beneath the epidermis, constituting up to 43% of the positively staining cells (100 nuclei). The distribution of the γ/δ T cells in different subsets is presented in Table 2 and illustrated in Figure 2. The dermal infiltrate consisted mainly of a mixture of CD4$^+$ and CD8$^+$ cells. The CD4/CD8 ratio was 1·5. TRC2$^+$ (α/β) cells constituted 48% of the positively staining dermal cells (100 nuclei). The periphery of the dermal infiltrate showed some CD1a$^+$ cells. The number of CD1a$^+$ epidermal cells was normal (4–8/0·3 mm). Keratinocytes expressed HLA-DR, although the majority of the dermal lymphocytes did not.

4H3$^+$ cells constituted 18% of the dermal infiltrate, but these cells were not present in the epidermis.

No significant difference was found between the number of Leu 4$^+$ cells stained with the two different staining methods.

Biopsy 2. The epidermal CD3$^+$ cell infiltration was slightly less marked than in the first biopsy. TCR1$^+$ cells were distributed very sparsely in the epidermis and dermis. The dermal infiltrate was a mixture of CD4$^+$ and CD8$^+$ cells with a CD4/CD8 ratio of 1·6. TCR2$^+$ (α/β) cells constituted 54% of the positively staining dermal cells (100 nuclei). Although an increase in oligoclonal 4H3$^+$ cells was detected in the peripheral blood, the percentage of 4H3$^+$ cells in the skin was found to be lower than in the first biopsy.

Biopsy 3. Immunophenotyping the inflammatory infiltrate yielded results comparable with those of the second biopsy. There were no γ/δ T cells present.

Discussion

A complete deficiency of either the enzyme adenosine deaminase (ADA) or purine nucleoside phosphorylase exists in a subgroup of patients with SCID. Whether the 5′-nucleotidase deficiency found in our patient played a role in his immune dysfunction, is not clear. A deficiency of 5′-nucleotidase has been described in some cases of Omenn's syndrome.[14] Although our patient had some features consistent with this syndrome (progressive course, early extensive erythroderma-like cutaneous lesions and alopecia), blood and tissue eosinophilia were lacking, making the diagnosis of Omenn's syndrome unlikely. One of the clinical signs which can occur early in the course of SCID is a *Candida* infection of the skin. Although a *Candida* infection was found in the first few months of life in this patient, there was no subsequent evidence of candidiasis.

The presence of so many TCR1$^+$ cells in the first biopsy was an unexpected finding, because these cells have been reported to be absent in mice with SCID.[15] It has been suggested that TCR1$^+$ T cells are specialized for epithelial defence.[16–18] In rodents, γ/δ TCR$^+$ T cells are preferentially present in epithelium, but in human epithelia the percentage of T cells expressing γ/δ is comparable with that observed in the peripheral blood.[7,19] These cells have been observed to be increased in infectious diseases in humans (localized cutaneous leishmaniasis; reversal reactions in leprosy; tuberculous lymphadenitis; in the peripheral blood of patients with measles) and in the gut epithelium of untreated coeliac disease.[19] Furthermore, studies of human neonatal skin have not demonstrated higher percentages of these cells than in adults (T.Vroom, personal communication). γ/δ T cells have never been described in SCID skin lesions. The current case is unique because of the presence of high numbers of T cells expressing TCR1 (γ/δ) in the skin, a finding which has not been described previously. Moreover, the presence of so many TCR1$^+$ cells was not consistent with the low numbers of these cells in the peripheral blood, suggesting that the cellular make-up of the blood need not parallel the cellular infiltrate in the skin. Whether the presence of a skin infection with *Candida albicans* during the first few weeks of life caused the appearance of γ/δ T cells in the skin is not clear. However, at the time of the first biopsy there was no evidence of *Candida* infection on the skin.

Oligoclonal T-cell proliferations have been described in SCID,[20] and TCR1$^+$ clonal epidermotropic T-cell proliferation was considered. The natural history of our patient, showing spontaneous disappearance of the TCR1$^+$ subpopulation is, however, not consistent with this hypothesis.

The difference in number of positively staining cells with different TCR1 specific MAbs, 11F2 and TCRδ-1 respectively, reflects the difference in reaction of the MAbs in acetone-fixed tissues.[7] The wide range in number of positively staining cells in different parts of the epidermis is in accordance with the observation that intraepidermal T cells occur in clusters.[21] In order to obtain information on the subsets of γ/δ T cells in the skin of our patient, we used the MAbs δTCS1 (recognizing cells expressing the Vδ1-Jδ1 encoded receptor,[8] Ti-γ-a (recognizing the Vδ9 encoded receptor)[9] and BB3 (recognizing the Vδ2 encoded receptor)[10] (Table 2). The normal differential has been reported to be 20% Vδ1-Jδ1 and 75% Vγ9 and/or Vδ2-positive cells[22] in the peripheral blood of children up to 6 months of age. In comparison with these data, the proportions were inverted in the skin of this patient, i.e. most of the cells expressed the Vδ1-Jδ1 repertoire. This finding suggests a preference for clones expressing the thymus-derived repertoire in the skin.[23] Because only 1% of peripheral T-lymphocytes were of TCR1 origin, no data were available from the peripheral blood of our patient.

Furthermore, the CD4/CD8 ratio in the skin was much higher than that in the peripheral blood, suggesting dermotropism of the CD4 lymphocyte subset. A higher CD4/CD8 ratio in the lesional skin than in the peripheral blood has also been found in atopic dermatitis.[24]

Interestingly, the increase in 4H3$^+$ T cells in the peripheral blood was not followed by an increase of this cell population in the skin. In healthy donors the antibody reacts with 5–10% of the peripheral blood T-cell population (K.C.Kuijpers *et al.*, unpublished data). In skin from healthy controls, 4H3$^+$ T cells were only present sporadically (unpublished data). In affected skin from patients with atopic dermatitis and psoriasis, no 4H3$^+$ T cells were found (unpublished data). The presence of these cells in the skin of our patient was, therefore, unusual. The small number of 4H3$^+$ cells *in situ* in comparison with the peripheral blood probably reflects the homing characteristics of these cells.

This case report illustrates that the use of monoclonal antibodies to identify cells involved in skin inflammation in SCID is a worthwhile technique in gaining better insight into the characteristics of the cells involved.

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British Journal of Dermatology (1992) 127, 286–288.

Granulomatous hypersensitivity to protamine as a complication of insulin therapy

M.M.HULSHOF, W.R.FABER, W.F.KNIESTEDT,* M.J.BOEREE† AND P.KREEK†
*Departments of Dermatology, *Pathology, and †Internal Medicine, Eemland Hospital, The Netherlands*

Accepted for publication 28 February 1992

Summary A patient is described who developed a granulomatous skin reaction to injections of protamine-insulin for the treatment of her diabetes mellitus. By means of skin testing it was shown that this reaction was caused by protamine. No adverse effects were seen when she was injected with insulin which did not contain protamine.

Patients with insulin-dependent diabetes mellitus may develop allergic reactions to insulin, protamine or other constituents of the insulin preparations. Usually, these are manifest as skin reactions at the injection sites. In such cases control of the diabetes may be difficult. Fortunately, as a result of improved manufacturing methods and the production of insulin of greater purity, allergic reactions are now less common.[1] Several different types of reaction have been described including early and late local reactions to human, pork and beef insulin,[2] flushing, pruritus, urticaria and angioedema due to protamine,[3] chronic urticaria during insulin therapy,[1] and zinc-induced granuloma.[4]

Case report

A 78-year-old woman with insulin-dependent diabetes mellitus was admitted to hospital because of difficulty in controlling her blood glucose levels. In addition, she had lesions at the sites of insulin injections on her abdominal skin which had appeared after she started treatment with Protophane HM insulin 3 years previously. A few minutes after each insulin injection a red macule, which changed into a papule and sometimes a pustule, developed at each injection site. These lesions persisted for at least 1 month. She had no previous history of skin disease, was not known to have any allergies (especially not to fish), and had not undergone vascular surgery.

On examination there were many red and purple-coloured macules and papules, and a few pustules and crusts, on the abdominal skin (Fig. 1).

Routine laboratory investigations, including full blood count, ESR, renal and liver function tests, were all normal. The blood sugar estimations ranged between 10 and 20 mmol/l. HbA1C was 14%. Bacterial culture from a pustule was negative. A biopsy of a papule showed several epithelioid cell granulomas, with foreign-body giant cells, in the dermis and subcutaneous tissue (Figs 2 and 3). Central necrosis was seen in some of the granulomas. Examination with polarized light was negative.

A hypersensitivity reaction to insulin, protamine or components of the insulin solution was suspected, and the following tests were performed.

1. Intracutaneous injection with two protamine-containing insulin preparations (Humulin NPH and Protophane NPH) and two insulin preparations which do not contain protamine (Humulin regular and Actrapid): after 2 days red papules developed at the sites of injection with the protamine-containing insulin preparations, and subsequently persisted for more than 4 weeks.
2. Intracutaneous injection with human Actrapid, porcine Actrapid, bovine Actrapid, diluting medium for Monotard, Actrapid, and Protophane, protamine sulphate and histamine 0·01% (NOVO skin testing set). An immediate-type reaction to histamine and protamine sulphate occurred, and at the site of protamine sulphate injection a red papule developed which persisted for more than 4 weeks. A biopsy, taken 3 weeks after injection of protamine sulphate, showed histological changes which were identical to those of the original lesions on the abdominal skin.

Correspondence: Dr W.R.Faber, Eemland Hospital, Location De Lichtenberg, Department of Dermatology, Utrechtseweg 160, 3818 ES Amersfoort, The Netherlands.

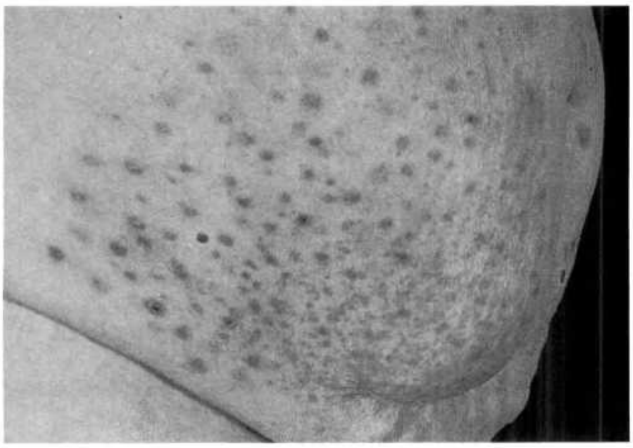

Figure 1. Disseminated macules and papules on the abdominal skin.

Insulin therapy was changed to a preparation which does not contain protamine sulphate (Monotard). Good control of the blood sugar was achieved and no further skin lesions developed.

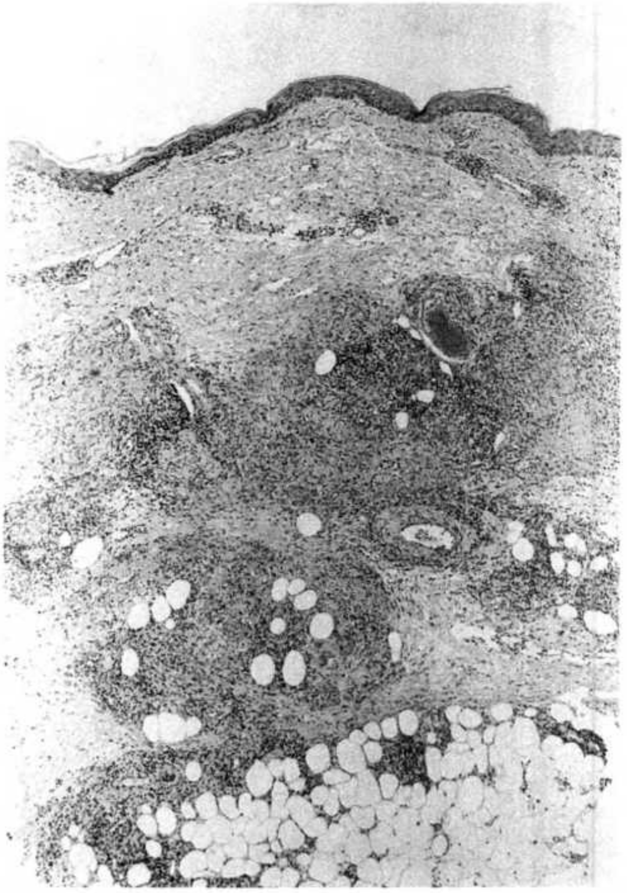

Figure 2. Granulomatous infiltrate extending into the subcutaneous tissue (haematoxylin and eosin × 100).

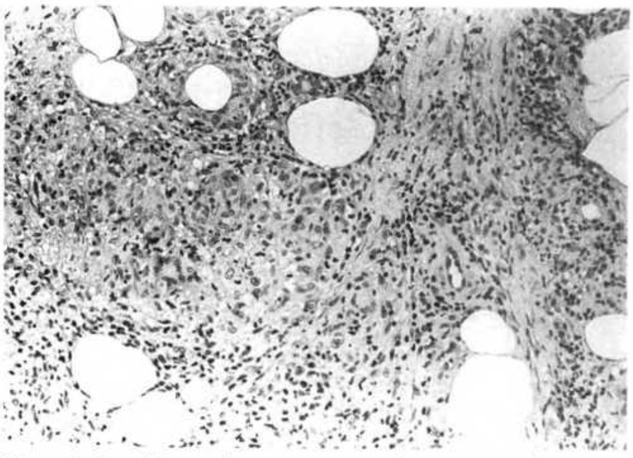

Figure 3. Detail from subcutaneous area showing granulomas with epithelioid cells, some giant cells and an admixture of lymphocytes (haematoxylin and eosin × 250).

Discussion

Protamine sulphate is a strong cationic polypeptide which is isolated from the testes of salmon and mammals, including man.[5] It is regularly administered after cardiac catheterization, cardiothoracic and vascular surgery, and (less frequently) after dialysis and leucophoresis, because of its capacity to reverse the anticoagulant activity of heparin.[6,7] Because protamine delays the absorption of insulin, it is combined with insulin in protamine zinc insulin (PZI) and neutral hagedorn insulin (NPH).[8] Because proteolytic enzymes must break down the protamine before the insulin can be released from the injection site, absorption is delayed and a prolonged duration of action is achieved.[1]

Adverse reactions to protamine occur infrequently, with an overall incidence of 0·13%.[9] Various reactions to protamine are described below.

Immediate-type hypersensitivity reactions

These are manifest by anaphylaxis, urticaria and flushing. They are presumably mediated by IgE-antibodies to protamine bound to mast cells.[1,10] Affected patients show a positive immediate-type reaction on intracutaneous testing with protamine. They have previously been exposed to protamine in vascular surgery, dialysis or insulin therapy, have undergone vasectomy or are known to have fish allergy.[11] In the last two examples there is a cross-reaction with protamine. Diabetic patients receiving daily subcutaneous injections with insulin containing protamine appear to have a 40–50-fold increase in the risk of life-threatening reactions when given protamine intravenously.[9,12]

It has been shown that IgE-antibodies may develop in patients with protamine-insulin-dependent diabetes which can result in the release of mediators from mast cells, basophils, or both, as a result of an immediate-type hypersensitivity reaction.[13] Protamine-binding IgE has been found in the sera of 53% of NPH treated diabetic subjects.[14] In patients who had an adverse reaction to intravenous protamine, and who had not been treated with subcutaneous protamine-insulin injections, IgG antibodies were found in the serum. This may be due to previous exposure to protamine, for example during vascular surgery.[15]

These facts support the hypothesis that humorally mediated responses to protamine are the likely cause of adverse reactions to intravenous protamine in protamine-insulin-dependent diabetics. Unfortunately, we did not have the opportunity to measure IgE and IgG antibodies in our patient.

Complement-mediated reactions

It has been suggested that the complement system can be activated by a protamine–heparin complex or by an interaction between protamine and complement-fixing IgG antibodies.[3] Protamine–heparin complexes have been shown *in vitro* and *in vivo* to activate complement through the classical pathway with the subsequent generation of anaphylotoxins C3d, C4a and C5a.[16,17] A patient has been described in whom an anaphylactoid reaction took place following the intravenous administration of protamine, and in whom complement activation was demonstrated by means of serial determination of C3 and C4 and split products C3c and C3d.[17]

In our patient it is apparent that the granulomatous reaction was due to protamine. It has been reported that in cases of insulin allergy or immunological resistance protamine was the only antigen recognized by T cells.[18] This suggests that in our patient protamine behaved as a persisting antigen giving rise to a granulomatous hypersensitivity reaction.[19] The granulomatous hypersensitivity reaction is a cell-mediated response to foreign antigen which results in epithelioid cell granuloma formation. It has been suggested that the initiating factor of organized granuloma formation might be T-cell-independent, and possibly a macrophage product,[20] while granuloma formation, once begun, is amplified by other factors such as T-cell-mediated immune responses.[21]

As far as we are aware, a granulomatous reaction to protamine has not previously been reported.

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British Journal of Dermatology (1992) 127, 289–291.

Pemphigus vegetans in a child

J.H.SILLEVIS SMITT, T.J.MULDER,* F.W.ALBEDA†‡ AND J.C.VAN NIEROP*

*Departments of Dermatology, *Paediatrics, and †Pathology, University of Amsterdam, Academisch Medisch Centrum, Emma Kinderziekenhuis/Het Kinder AMC, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands*

Accepted for publication 16 April 1992

Summary Pemphigus vegetans was diagnosed in a 12-year-old boy based on clinical, histopathological and immunohistological findings. To our knowledge, this is the first case of juvenile pemphigus vegetans to be published in the literature. Suggested treatment with prednisone and azathioprine was refused, and the patient was treated with a decoction of herbs prescribed by a practitioner of traditional Chinese herbal medicine. This treatment gave excellent results. Possible active components of the treatment are discussed.

Pemphigus is very rare in children, although endemic pemphigus, fogo selvagem, occurs quite frequently in children in endemic areas.[1] Pemphigus foliaceus[2] and pemphigus vulgaris[3] are known to occur in childhood, and 35 children with pemphigus vulgaris have been reported in the literature. The clinical picture and the prognosis in children are comparable with the disease in adults.[3] Although pemphigus vegetans is mentioned in textbooks of paediatric dermatology,[4,5] no reference is made in these books to published case reports. We present a case of juvenile pemphigus vegetans and note the surprisingly good result from treatment with a herb decoction prescribed by a practitioner of traditional Chinese medicine.

Case report

A 12-year-old Chinese boy was referred to our hospital with a 5-month history of stomatitis. Initially, painful blisters appeared on his tongue and on the inside of his lips, and subsequently he gradually developed a severe stomatitis. Although there were some episodes of fever, his general condition remained good. Treatment with analgesics and amoxycillin was unsuccessful and the boy was referred to our hospital. On presentation the inflammation had spread to the perioral skin, and skin lesions had also appeared on other parts of the body. The patient complained of an extremely painful mouth and throat, and of chest pain. In spite of a good appetite, his food intake had diminished. There was a loss of weight of 4 kg in 2 weeks.

Physical examination on admission showed a slender boy, height 144 cm, weight 32 kg, temperature 37·4°C, who could hardly speak due to painful erosions and swelling of the lips and tongue. There were erythematous papular lesions with crusts and some small blisters on the nares (Fig. 1), the perioral skin, the axillae, the groins and the umbilical and perianal regions. In addition there was a balanitis. There was no lymphadenopathy.

Investigations showed an ESR of 5 mm/h; white cell count $8{\cdot}5 \times 10^9$/l, with a normal differential; eosinophils 490×10^6/l. *Staphylococcus aureus* was isolated from the skin lesions. Histopathology of the first skin biopsy (Fig. 2) showed erosion of the epidermis, with a purulent exudate. Some necrosis of keratinocytes was seen, while the nuclei of the basal cell layer showed hypochromasia and eosinophilic nucleoli. There was minimal focal suprabasal acantholysis. Because an extensive mixed cellular infiltrate was seen in the dermis, with neutrophils in the epidermis, the lesions were interpreted as being of infectious origin. Serological investigations suggested a recent herpes simplex virus (HSV) infection, which was compatible with the histopathology showing cytonuclear changes suggestive of a viral infection.

He was treated with oral acyclovir (200 mg 5 times/day) for 3 weeks. During this period the HSV-antibody titre decreased. There was no serological evidence of other viral or bacterial infections. Immunological investigations showed no evidence of immunodeficiency. The skin lesions, especially those in the axillae and groins,

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Correspondence: Dr J.H.Sillevis Smitt, Department of Dermatology, Academisch Medisch Centrum, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

‡ Deceased.

This case was presented, in part, at the 6th International Congress of Pediatric Dermatology, 8–11 June 1992, Toronto, Canada

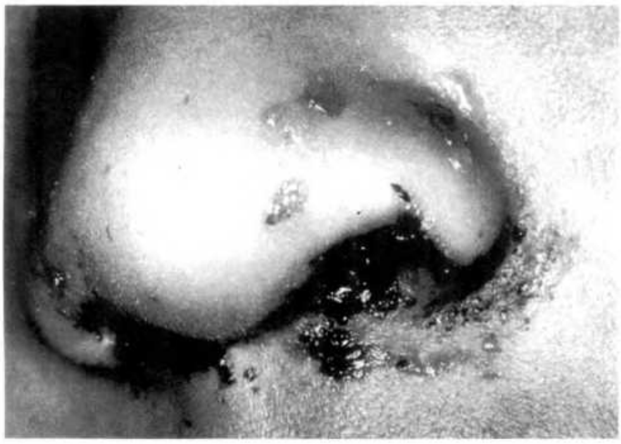

Figure 1. Some small blisters on the nares and haemorrhagic crusts on skin and mucous membrane of the nose.

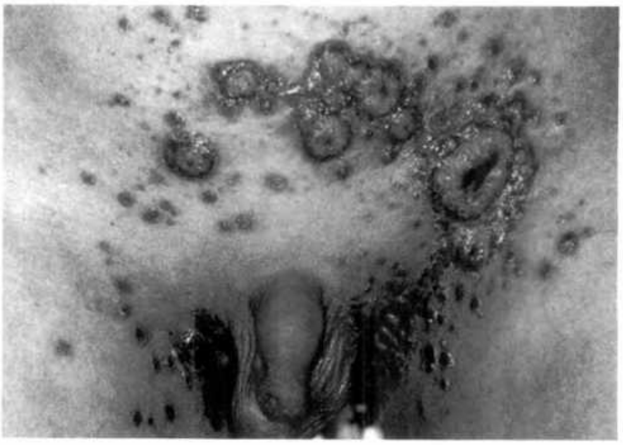

Figure 3. Large vegetating, erosive plaques in combination with some small blisters in the groins.

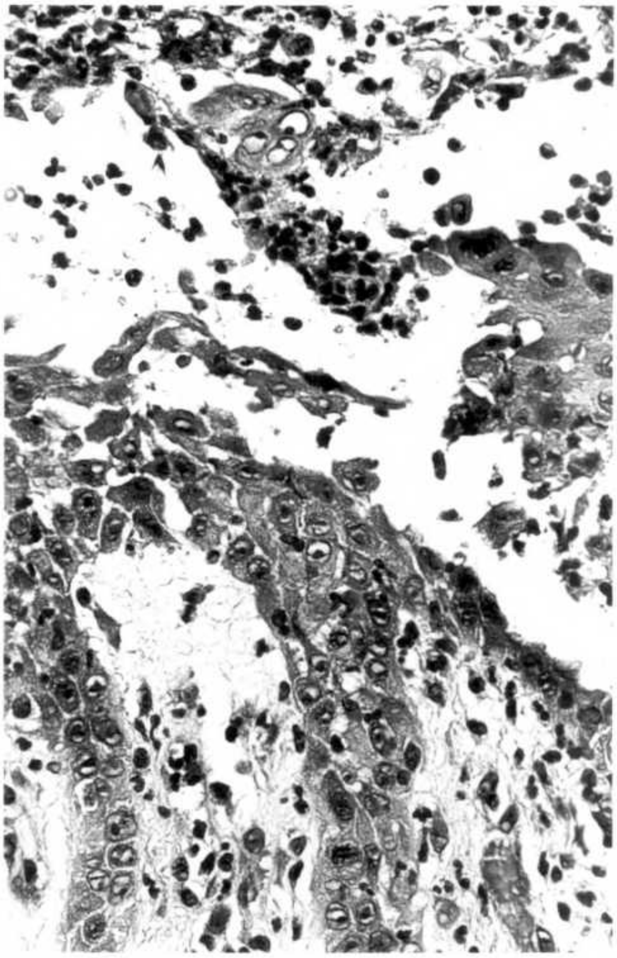

Figure 2. First skin biopsy showing partial erosion of the epidermis, with a purulent exudate and keratinocyte changes consisting of nuclear hypochromasia, large eosinophilic nucleoli and focal multinucleate keratinocytes (haematoxylin and eosin, × 200).

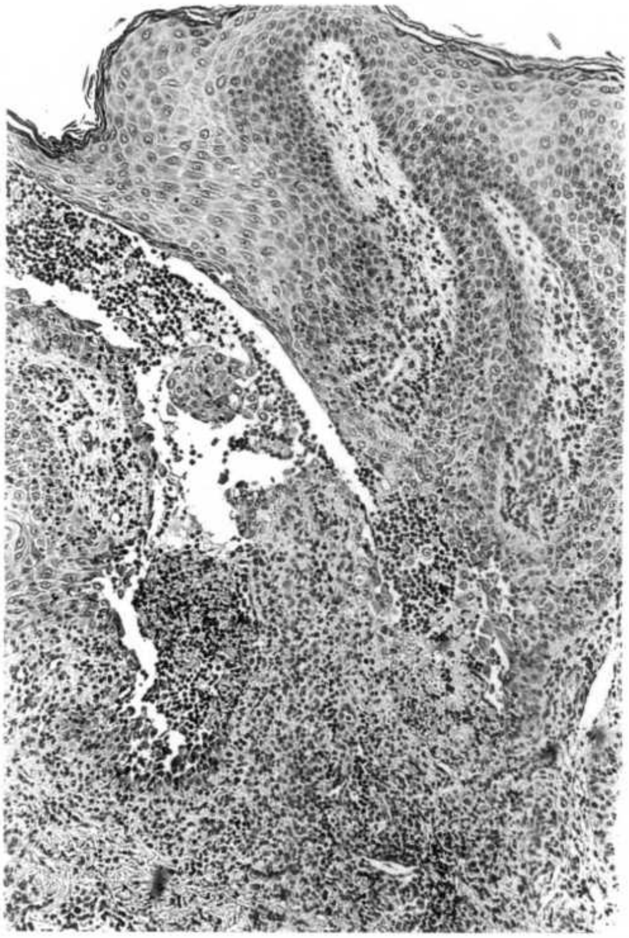

Figure 4. Second skin biopsy showing a hyperplastic epidermis with suprabasal acantholytic cleft formation and extensive infiltration by eosinophils (haematoxylin and eosin × 125).

grew into large, very painful, vegetating plaques (Fig. 3). Histopathology of one of these lesions showed a papillomatous, hyperplastic epidermis with suprabasal acantholysis. Some of the suprabasal clefts were filled with eosinophils. The histological picture was diagnostic of pemphigus vegetans (Fig. 4).

Direct immunofluorescence of the biopsy showed the presence of intercellular IgG antibodies, characteristic of pemphigus. The serum titre of the antibodies against intercellular epidermal antigen was 1:240. A diagnosis of pemphigus vegetans was made, and treatment with prednisone in combination with azathioprine was proposed. However, the boy's parents refused this treatment and turned to a practitioner of traditional Chinese herbal medicine, who treated him with a decoction consisting of a mixture of herbs (containing *Taraxacum mongolicum* [Hand.-Mazz.], *Viola yezoënsis* [Maxim.], *Cortex rhizoma*, *Paeonia albiflora* [Pall.], *Paeonia Moutan* [Sims.], *Glycyrrhiza uralensis* [Fisch.], *Polyporus cocos* [Schw.], *Scutellaria rivularis* [Wall.], *Oldenlandia diffusa* [Roxb.] and *Smilax glabra* [Roxb.], as its constant components). This treatment resulted in complete clearance of skin lesions, leaving only post-inflammatory hyperpigmentation. The oral lesions did not disappear completely. A fasting morning serum cortisol taken during treatment with this decoction was within normal limits. At present, 1 year after commencing treatment with the herb decoction, the patient is still in remission, although the antibody titre against intercellular epidermal antigen is unchanged.

Discussion

Pemphigus vegetans is considered to be a pemphigus variant, ultimately often evolving into pemphigus vulgaris. At first presentation most patients show stomatitis, as did our patient. The herpes simplex infection, leading to blisters showing acantholysis, caused delay in diagnosis in our case. The question arises as to whether the herpes infection provoked the onset of the pemphigus.

Good therapeutic results are obtained in the treatment of pemphigus with prednisone and azathioprine, but these agents may give rise to significant side-effects. Our patient was by chance treated with a decoction of herbs, prescribed by a practitioner in traditional Chinese medicine. To obtain an indication of the possible presence of corticosteroids in this decoction, a fasting serum cortisol was performed, and showed a value within normal limits. This suggests that no, or at least no considerable amount of, corticosteroids was present in the decoction.

Recently the effectiveness of Chinese herbs in the treatment of atopic eczema has been reported.[6] It has been suggested that paeonol, present in various plants including those of the genus *Paeonia*, was an active component of this preparation in the treatment of eczema.[7,8] Our patient's herb decoction contained two plants of the genus *Paeonia*. Whether paeonol is the effective component in the treatment given to our patient, remains to be determined.

Acknowledgments

We would like to thank Mr Fong Chi Kwan and Mrs Yvonne Lau Shuet Ming, for making the composition of the decoction available, and Ing W.N.J.Ursem, Hortus Botanicus, Amsterdam, for botanical advice.

Addendum

To our deepest regret our colleague F.W.Albeda died on 27 February 1992.

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British Journal of Dermatology (1992) 127, 292–294.

Verapamil-induced secondary erythermalgia

J.P.H.DRENTH,*§ J.J.MICHIELS,* T.VAN JOOST† AND V.D.VUZEVSKI‡

Departments of *Haematology, †Dermatology, and ‡Clinical Pathology, University Hospital, Erasmus University Rotterdam, The Netherlands

Accepted for publication 15 April 1992

Summary A 59-year-old man developed red, swollen and warm feet accompanied by intermittent burning pain during treatment for cardiac failure and arrhythmias with several drugs including verapamil. The condition gradually worsened until there was persistent disabling burning pain and severe erythema and swelling of the feet. Aspirin and other analgesics were ineffective in relieving the discomfort. Histopathology of punch biopsies showed a mild perivascular mononuclear infiltrate and moderate perivascular oedema. Within 2 weeks of stopping verapamil the burning pain, erythema, and swelling of the feet had resolved. The clinical features and subsequent course are consistent with a diagnosis of erythermalgia secondary to verapamil.

Recurrent attacks of severe burning pain, erythema, warmth and swelling of the extremities are designated either as erythromelalgia, or as primary or secondary erythermalgia.[1] Erythromelalgia is invariably associated with thrombocythaemia. The clinical features are the result of platelet-mediated arteriolar inflammation and thrombosis. Aspirin alleviates the symptoms by irreversibly inhibiting platelet cyclo-oxygenase activity and platelet aggregation.[2–4] The idiopathic variant of burning and painful extremities is designated primary erythermalgia. It occurs spontaneously in childhood or adolescence in the absence of any detectable underlying disorder.[5,6] It typically presents as bilateral, symmetrical burning pain associated with erythema of the feet, ankles and legs. The symptoms are exacerbated by heat and exercise, and usually deteriorate to such a degree that the patient is compelled to lead a sedentary life-style and continuously attempts to find a means of cooling the feet.

Secondary erythermalgia is defined as an acquired, persistent, but potentially curable, symmetrical, painful erythema of the extremities.[4–6] It occurs as an adverse effect of several drugs, including the ergot derivatives pergolide and bromocriptine,[7–10] and the vasodilating calcium antagonists nifedipine and nicardipine.[11–15] In this report we present a typical case of acquired secondary erythermalgia occurring in a patient receiving long-term treatment with verapamil.

Case report

A 59-year-old man had a history of myocardial infarction in 1984 and a coronary artery bypass operation, with resection of a left ventricular aneurysm, in 1987. Since March 1990 he had been treated for atrial fibrillation and cardiac failure with a number of drugs including daily digoxin 0·25 mg, frusemide 80 mg, spironolactone 25 mg, captopril 25 mg, verapamil 120 mg and a coumarin derivative. A few months after starting this treatment he complained of intermittent redness, warmth and swelling of the feet, together with burning pain, occurring more often during warm weather and in the evening. The symptoms gradually worsened and were eventually present constantly throughout the day and night. The unbearable burning pain could only be relieved by immersing his feet in ice-cold water. Aspirin and many other analgesics provided no relief. When we saw him in February 1991 the feet and ankles were symmetrically swollen and erythematous, and were extremely tender (Fig. 1). The peripheral pulses were palpable.

A full blood count, electrolytes and urea, and liver function tests were normal. Antinuclear antibodies, rheumatoid factor and an autoantibody screen were negative. Histology of punch biopsies from the affected areas showed slight to moderate perivascular oedema, and a mild inflammatory infiltrate around capillaries in the upper dermis. The capillary walls appeared to be

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Correspondence: Dr J.J.Michiels, University Hospital Dijkzigt, Department of Haematology, Dr Molenwaterplein 40, 3015 GD Rotterdam, The Netherlands.

§Present address: University Hospital St Radboud, Department of Medicine, Division of General Internal Medicine, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

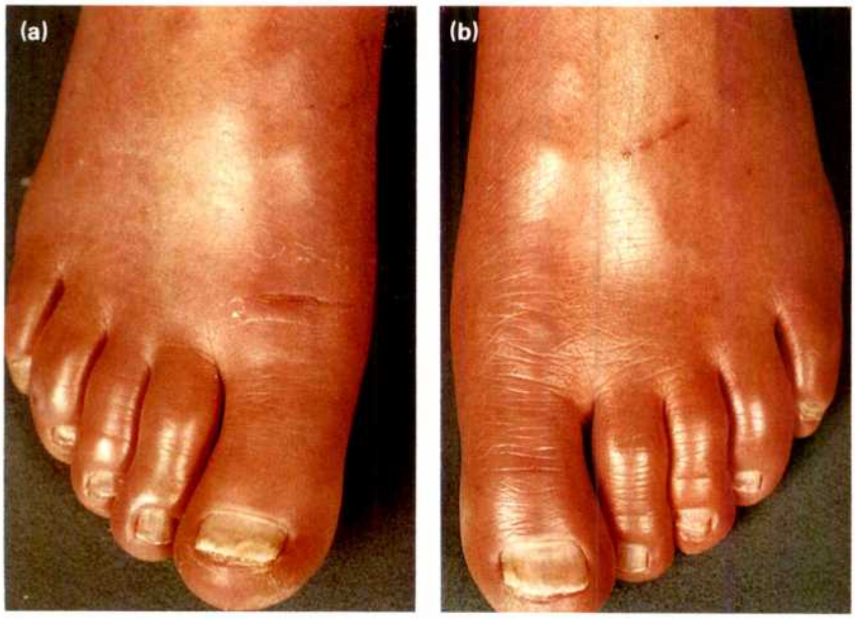

Figure 1. Bilateral, symmetrical erythema and swelling of the feet and ankles.

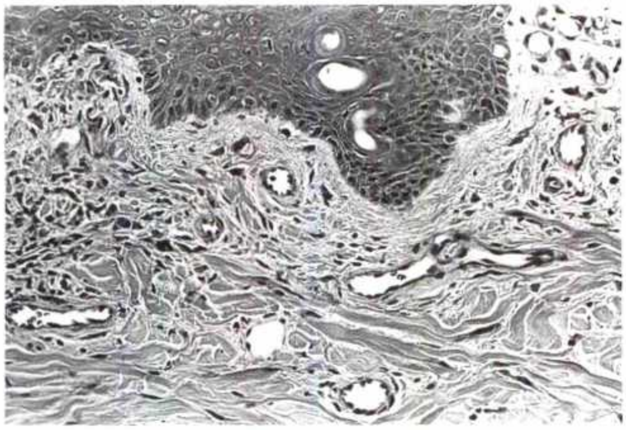

Figure 2. Photomicrograph showing a mild pericapillary inflammatory infiltrate and oedema, in the upper dermis. There is mild to moderate swelling of endothelial cells (haematoxylin and eosin × 120).

slightly thickened, and there was mild to moderate swelling of endothelial cells (Fig. 2). Because calcium antagonists are known to cause secondary erythermalgia,[11–15] treatment with verapamil was stopped. His symptoms gradually resolved over the course of 1–2 weeks. The temporal relation with verapamil, and the absence of other causes, make this drug the most probable cause of the erythermalgia. The patient refused rechallenge with verapamil.

Discussion

Drug-induced secondary erythermalgia has been described in association with the long-term use of ergot derivatives and calcium antagonists (Table 1). Bromocriptine has been reported to induce erythermalgia-like changes, and in two large studies of patients with parkinsonism 18 of 202 patients developed red, tender, warm, oedematous feet, ankles and lower legs. Some discomfort, described as a burning sensation, was noted in most cases.[7,8]

The ergot derivative pergolide, a drug used to control the tremor of Parkinson's disease, has also been reported as causing an erythermalgia-like eruption in two cases.[10] Both patients suffered from a bilateral symmetrical erythema of the skin. Biopsy in one patient showed marked focal capillary proliferation in the upper dermis and a minimal inflammatory infiltrate.

Three studies report a connection between the calcium antagonist nifedipine and incapacitating secondary erythermalgia.[11–13] The patients complained of burning pain, and had symmetrical erythema of the feet and lower legs which subsided within 2 days of withdrawal of the drug. Resumption of the drug resulted in recurrence of the symptoms.

The calcium antagonist nicardipine has been reported as causing intermittent redness, warmth and pain in the fingers of one patient.[14] These changes rapidly resolved after discontinuation of the drug.

As we observed in our case, erythermalgia secondary to drugs develops insidiously after long-term treatment, and disappears 1–14 days after the responsible drug is stopped.

Our patient presented with typical erythermalgia of both feet and lower legs. The regression of the erythér-

Table 1. Drug-induced secondary erythermalgia

Drug	Dose (mg/day)	*n*	*T*1	*T*2	Reference
Bromocriptine	115	7/92	10 months	1 week	Calne *et al.*[7]
Bromocriptine	101	11/110	13 months	10 days	Eisler *et al.*[8]
Bromocriptine	120	1	24 months	NS	Dupont *et al.*[9]
Pergolide	NS	1	12 months	NS	Monk *et al.*[10]
Pergolide	NS	1	18 months	NS	Monk *et al.*[10]
Nifedipine	60	1	8 weeks	2 days	Fisher *et al.*[11]
Nifedipine	40	1	NS	Immediate	Brodmerkel[12]
Nicardipine	60	1	1 year	Rapid	Levesque *et al.*[14]
Verapamil	120	1	1–9 months	1–2 weeks	Present case

n, number of patients; *T*1, time lapse between first dose and occurrence of erythermalgia; *T*2, time from discontinuation to resolution of erythermalgia; NS, not stated.

malgia after stopping verapamil strongly suggests that it was responsible for the problem. Unfortunately, the patient refused rechallenge with verapamil and absolute confirmation was therefore not possible. However, the temporal relationship with verapamil makes this drug the most likely cause of the erythermalgia.

Verapamil is a phenylalkylamine derivative able to inhibit the slow inward current of calcium ions across the cell membrane of cardiac and vascular smooth muscle. It thereby causes coronary and peripheral vasodilatation and decreases myocardial contractility. It also influences the sinus and atrioventricular nodes. Verapamil is indicated for the treatment of hypertension, cardiac arrhythmias and angina.[16] A common side-effect of verapamil is facial flushing and this is regarded as an extension of the vasodilator action.[16]

To our knowledge, our case is the first report of verapamil-induced secondary erythermalgia. The pathophysiology of primary and secondary erythermalgia and the mechanism of drug-induced erythermalgia are unknown and remain to be elucidated.[6]

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British Journal of Dermatology (1992) 127, 295–296.

White fibrous papulosis of the neck

R.K.JOSHI, A.ABANMI AND A.HALEEM
Riyadh Armed Forces Hospital, Riyadh, Saudi Arabia

Accepted for publication 5 March 1992

Summary We report two middle-aged women who presented with multiple, discrete, non-follicular, oval, pale, asymptomatic lesions on the neck. Clinical and histopathological features were compatible with the entity of white fibrous papulosis of the neck.

The recently recognized entity of white fibrous papulosis of the neck was first described by Shimizu *et al.* in 1985.[1] Disorders such as pseudoxanthoma elasticum, cutis laxa, eruptive vellus hair cysts, milia, eruptive xanthoma and perifollicular elastolysis may be considered in the differential diagnosis because of the morphology of the lesions and their location, but white fibrous papulosis can be distinguished from these by the age of onset and histological features.

All the originally reported cases of this condition were Japanese,[1,2] but in 1991 Cerio *et al.*[3] published an account of an Iranian patient with white fibrous papulosis. We report two Saudi women with this dermatosis.

Case reports

Case 1

This Saudi woman in her early sixties presented with a history of skin lesions on her neck of 2 years duration. The lesions were asymptomatic and had gradually increased in number. She was known to suffer from diabetes mellitus, hypertension and duodenal ulcer, and was receiving treatment with metformin, atenolol, domperidone and natrilix. There was no family history of a similar skin condition.

On examination the skin of her neck was wrinkled, and there were numerous (80–100) firm, round to oval, hypopigmented, non-follicular papules, 1–3 mm in size, on the sides and nape of the neck (Fig. 1). The appearance was suggestive of pseudoxanthoma elasticum. However, there were no angioid streaks on ophthalmological examination. Histology of a skin biopsy showed some thickening of the collagen bundles and paucity of elastic fibres in the mid-dermis (Figs 2 and 3).

Case 2

A 60-year-old Saudi woman complained of skin lesions on the neck of over 7 years duration.

On examination the skin on the neck was thin and wrinkled, and there were approximately 100 papules on the sides and back of the neck. These papules were 1–3 mm in size, hypopigmented, oval to round, and non-follicular. There were no angioid streaks on ophthalmological examination. Histology of a biopsy of one of the papules showed changes similar to those in Case 1.

Discussion

There have been very few reports of this disorder since the original description of 16 patients by Shimizu *et al.* in 1985. Thirty-two cases of white fibrous papulosis were subsequently reviewed by the same authors.[2] Apart from a relatively circumscribed area of thickened collagen bundles in the papillary and mid-dermis, and slight paucity of elastic tissue in 10 of 25 specimens, no other specific features were found on light microscopic and ultrastructural examination. Our patients also showed similar changes, which could be responsible for the wrinkled appearance of the skin. Eighty-one per cent of the 32 patients reported in Japan were men. Their ages ranged from 39 to 79 years, with a mean of 62 years. The papules varied between 10–100 in number and 1–3 mm in size.

Both our cases exhibit the clinical and histological features of this condition. We agree with Shimizu *et al.*[1,2] that it is a distinct clinicopathological entity. Its aetiology is unknown, but in view of the onset late in life, it is likely to be the result of an age-related change in collagen structure. There is no evidence of familial occurrence of this abnormality, or that it is a cutaneous indicator of systemic disease.

The patient described by Cerio *et al.*[3] was a 70-year-old female who was also from the Middle East. It is possible

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Correspondence: Dr R.K.Joshi, Consultant Dermatologist, Riyadh Armed Forces Hospital, P.O. Box 7897, Riyadh 11159, Saudi Arabia.

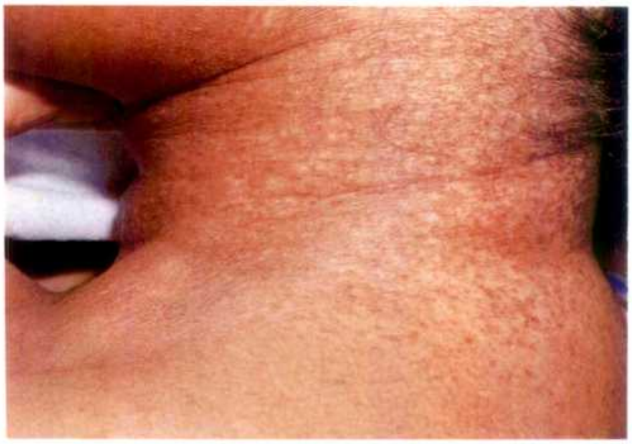

Figure 1. Multiple discrete papules and wrinkled skin on the side of the neck.

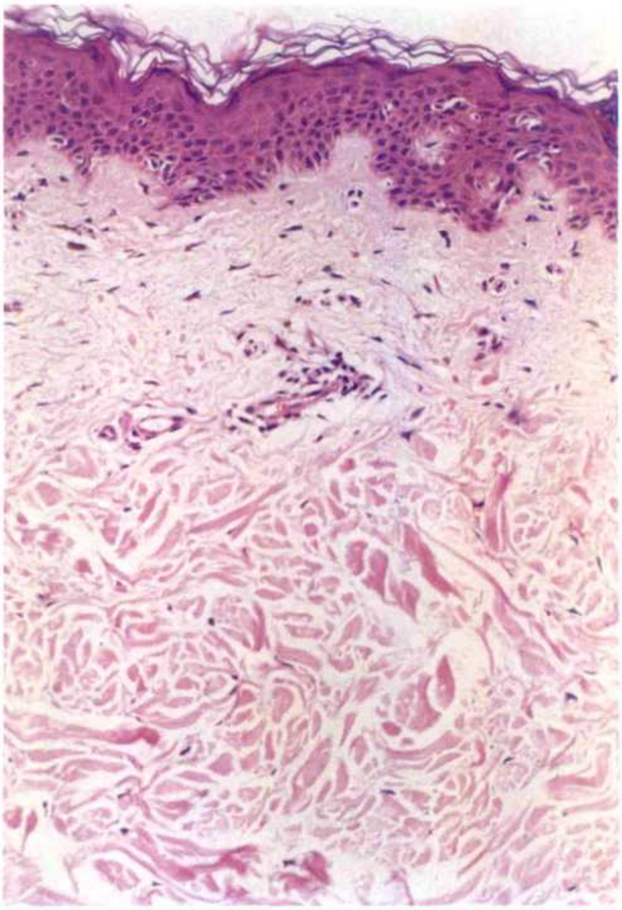

Figure 2. The epidermis and the papillary dermis are unremarkable. The mid-dermis shows thickening of the collagen bundles (haematoxylin and eosin × 150).

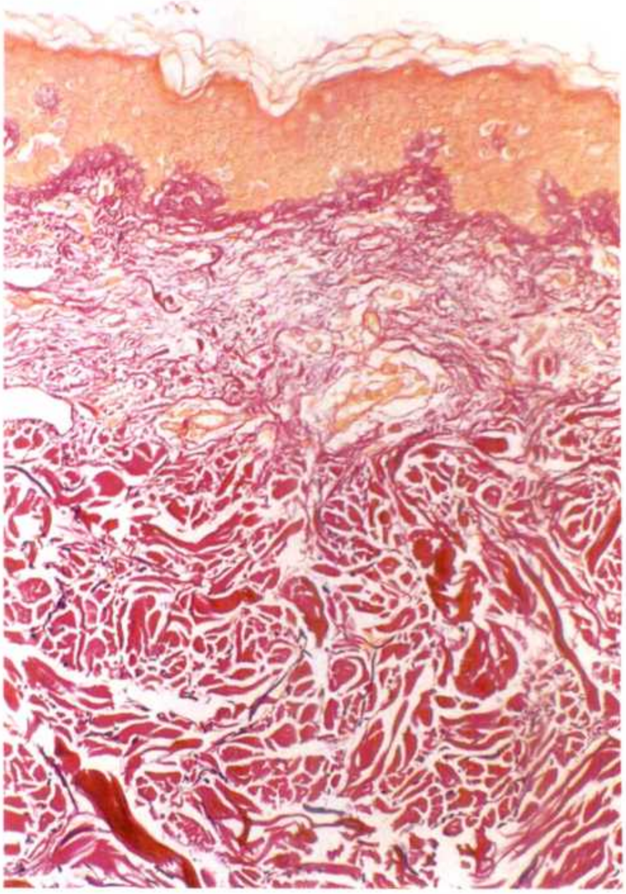

Figure 3. There is moderate paucity of elastic fibres in the mid-dermis (elastic Van Geeson × 150).

that this condition might be more common in females in the Middle East, in contrast with Japan where males are predominantly affected.

Because the lesions are asymptomatic, patients may not seek medical help, and this condition may be more common than the few case reports suggest. Greater awareness among dermatologists is likely to lead to this dermatosis being recognized more frequently.

As far as we are aware, this is the first report of this condition from Saudi Arabia.

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British Journal of Dermatology (1992) 127, 297–299.

WORKSHOP REPORT

Diagnostic phototesting in the United Kingdom

BRITISH PHOTODERMATOLOGY GROUP
Members of the Group who contributed to this report are: D.Bilsland, B.L.Diffey, P.M.Farr, J.Ferguson, N.K.Gibbs, J.L.M.Hawk, B.E.Johnson, I.A.Magnus, H.Moseley, G.M.Murphy, P.G.Norris, A.Pierce

Accepted for publication 30 April 1992

Phototesting is an essential part of the investigation of the photodermatoses, adding an objective basis for diagnosis to history and examination. A workshop organized by the British Photodermatology Group was held recently to discuss current uses, methodology and problems of phototesting. The workshop was designed to produce clarification of these points for the general dermatologist, as well as providing a forum for the critical examination of specific areas, including the possibilities for standardizing U.K. phototest procedures. Techniques and uses of photopatch testing were not discussed.

Phototesting aids diagnosis through demonstration of abnormal responses in terms of wavelength dependence, exposure dose, morphology and time-course of the reactions produced. Repeat phototesting allows the natural progression of a disease to be monitored, or its response to treatment. The phototesting techniques developed for diagnosis are also used for research and it is clear that interest in and developments of phototesting have resulted in advances in the understanding of the photodermatoses.

Routine diagnostic phototesting is a procedure which remains restricted to a few centres in the U.K. Unlike patch testing, which is largely qualitative, phototesting must be quantitative if clinically useful results are to be obtained. This requires appropriate knowledge of light sources and their measurement, and for this reason collaboration with a medical physicist experienced in photophysics is desirable.

The simplest and cheapest sources of ultraviolet and visible radiation are fluorescent lamps. The spectral emission from a fluorescent lamp is normally broad and as a consequence only crude information regarding wavelength sensitivity can be obtained. Fluorescent lamps with a significant UVB emission (e.g. Philips TL-12) can be used to provide information on erythemal sensitivity[1] with irradiation times of a few minutes. Unfortunately the exposure times using UVA fluorescent lamps are too long to allow a similar investigation of erythemal sensitivity to UVA radiation. Nevertheless if 10 or more UVA fluorescent lamps are mounted in an appropriate configuration sufficient intensity (~20 mW/cm^2) is available to provoke lesions over large areas (e.g. forearm and hand) with exposure times of 10–20 min. Other lamps which can be used for photoprovocation include optically filtered high-pressure metal halide lamps (e.g. UVASUN manufactured by Mutzhas in Germany). This type of lamp emits at high intensity (>50 mW/cm^2 at 30 cm from the lamp) in the wavelength interval 340–400 nm. High-pressure xenon arc lamps, optically filtered to produce an emission spectrum simulating that of terrestrial sunlight, can be used in the same way.

In order to determine the erythemal response of skin to different wavelengths of optical radiation (action spectroscopy) an irradiation monochromator, in which a diffraction grating or prism is employed to disperse light from a xenon arc lamp, provides discrete wavebands of ultraviolet and visible radiation. Proper use of an irradiation monochromator and the associated radiation detector (normally a thermopile) requires careful attention to quality assurance.

There is inconsistency amongst centres which investigate photodermatoses with regard to test site, wavebands and definition of a minimal erythema dose. This makes direct comparison of results difficult. Nevertheless, most centres will investigate responses to a number of wavebands within the ultraviolet region and often extending into the visible spectrum.[2–6]

There are normally two types of procedure in phototesting:

1. Determination of threshold responses mainly uses the minimal erythema dose (MED) or the minimal exposure dose required to induce an urticarial response in the case of solar urticaria. These tests are normally performed on the skin of the back which allows a large number (often more than 30) of test exposures. Threshold doses are usually determined at a number of different wavebands using a geometric series of exposures. Examples of

Correspondence: Dr J.Ferguson, Photobiology Unit, Department of Dermatology, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.

Table 1. Wavebands used to determine MED

Central wavelength (nm)	Bandwidth ± (nm)	Irradiance on patients' skin mW/cm^2	Average* MED (normal range) J/cm^2	Exposure time to achieve MED
300	5	6	0·034 (0·014–0·080)	6 s
305	5	2	0·060 (0·028–0·132)	30 s
320	10	25	2 (1–4)	1 min 20 s
330	15	60	7 (3·5–14)	2 min
335	30	95	5·25 (1·6–17·3)	55 s
350	30	180	28 (14–56)	3 min
365	30	122	14·8 (7·6–29)	2 min
400	30	220	80 (40–160)	6 min
430	30	114	>82	>12 min

*For 300, 320, 330, 350, 400 nm: median and normal range.
For 305, 335, 365 nm: geometric mean and 5% confidence limits.

wavebands used, with pertinent data, are shown in Table 1. Some centres carry out irradiation at a single visit and determine the MEDs by visual inspection 24 h later. Another approach is to perform irradiation with coarse dose increments on day 1 and note erythemal responses immediately after exposure, during the first hour and at about 6 and 24 h to give an indication of the reaction time course and an approximation of the MED. A second series of exposures is given on day 2 with finer dose increments centred around the approximate MED. However, there remains disagreement amongst centres as to whether examining the irradiation sites several times during the first 24 h improves diagnostic accuracy, except, of course, in those patients with solar urticaria in whom determination of minimal urticarial doses must be carried out within 10 min or so of irradiation. Once the MED has been determined in a particular waveband it must be compared with the normal range established by the relevant phototesting centre.[7]

A number of instruments which allow quantification

Table 2. The photosensitive disorders and results of phototesting

Diagnosis	Delayed (e.g. 24 h) erythemal responses	Other tests/comments
Polymorphic light eruption	Usually normal	Lesion provocation by repeated exposure of large area of skin
Photosensitivity dermatitis/actinic reticuloid syndrome Chronic actinic dermatitis	Abnormal to UVB, UVA and in some cases to visible wavelengths	
Actinic prurigo	Variable, may be normal in some cases	Lesion provocation by repeated exposure of large area of skin. Abnormal response of UV erythema to topical indomethacin
Solar urticaria	Abnormal erythemal responses to UVA/B or visible	Urticarial response to UVB, UVA or visible light, maximal within 30 min after irradiation
Hydroa vacciniforme	Usually normal	Lesion provocation by repeated exposure of large area of skin
Drug-induced photosensitivity	Usually abnormal to UVA and in some cases visible	Abnormal erythemal responses may occur soon after irradiation
Xeroderma pigmentosum Cutaneous porphyrias	Abnormal to UVB extending into UVA	Delayed in onset and persistent in duration

of erythemal responses are now available commercially. Although useful for research purposes, particularly in order to study the dose–response relationship of ultraviolet erythema,[8] these instruments are not used by any centre for routine diagnostic phototesting.

2. Phototesting in order to reproduce lesions typical of the photodermatosis in question is unlikely to be successful with an irradiation monochromator because exposure sites are usually 1 cm or less in diameter (the exceptions to this are photosensitivity dermatitis and solar urticaria). Instead, greater likelihood of success is achieved if a much larger area of skin is irradiated using an array of fluorescent lamps, or optically filtered metal halide or xenon arc lamps. Even here, results can often be negative with a single exposure, and successful provocation may require repeated exposures on several consecutive days to previously affected skin.

In a diagnostic sense, current phototesting procedures are helpful in several clinical situations (Table 2). A particular area of diagnostic importance is that of middle-aged to elderly men who develop dermatitis, maximal on, or confined to, exposed sites where photosensitivity is suspected. Because of the combination of contact allergy and photosensitivity dermatitis, phototesting frequently produces results which are unexpected on clinical grounds; in this situation a negative result may be as important as a positive response with regard to diagnosis and management.

The diagnostic criteria for actinic prurigo remain difficult,[9] particularly regarding its differentiation from polymorphic light eruption by phototest results.

Overall, it was concluded that phototesting, taking approximately 1 h per session, is an underused procedure helpful in the management of most photodermatoses and essential for diagnosis in some cases. The expertise required for a good phototesting programme is currently restricted to a small number of centres willing to accept patients for investigation. However, consultation concerning basic or more refined equipment and its use is readily available from individuals with expertise in photodermatology, or through the British Photodermatology Group.

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British Journal of Dermatology (1992) **127**, 300–301.

Correspondence

Relapsing polychondritis—treatment with cyclosporin A

Sir, The case report by Anstey *et al.*[1] was interesting and raises the question of the place of cyclosporin in the treatment of relapsing polychondritis. Because of the rarity of this disease, new information on treatment is only likely to be acquired through such case reports. We would like to add some information based on personal experience in treating a patient, and from the sparse literature on this subject.

We treated a 14-year-old girl with relapsing polychondritis who had severe disease involving the pinnae, nasal cartilage, inner ear, sclera, kidneys and trachea which was not controlled by azathioprine 150 mg/day in combination with prednisolone 30 mg/day. Because of progressive tracheal stenosis, treatment was changed to cyclosporin 5 mg/kg/day with improvement in all indicators of disease activity for 3 months. However, while on this dose of cyclosporin and prednisolone, the tracheal stenosis progressed, with collapse from 8 to 4 mm in diameter. Changing to cyclophosphamide 150 mg/day and prednisolone 50 mg/day arrested this progression, and has controlled the disease for 2 years in reduced dosage.[2]

Svenson's patient, referred to by Anstey *et al.*,[1] was described in more detail elsewhere[3] and also had life-threatening pulmonary disease with tracheal stenosis, despite treatment with azathioprine and subsequently with cyclophosphamide 100 mg/day and prednisolone 60 mg/day. She rapidly improved with cyclosporin 15 mg/kg/day and showed a fall in anti-collagen II antibodies with both cyclophosphamide and cyclosporin. She was eventually controlled on cyclosporin 6 mg/kg/day and her disease remained inactive 2 years later.

A further patient with tracheal stenosis was reported by Rogerson *et al.*[4] Their patient was treated with high-dose intravenous prednisolone, and steroid reduction was made possible by the addition of cyclosporin 6·25 mg/kg/day. The authors cite a further case showing a dramatic response to cyclosporin. A recently described patient with cardiac involvement responded well to cyclosporin.[5] However, one of the patients described by Lim and Chan with tracheal stenosis was unresponsive to cyclosporin and other immunosuppressants, including azathioprine and cyclophosphamide.[6] Finally, a patient who failed to respond to prednisolone (up to 60 mg/day) in combination with azathioprine 2·5 mg/kg/day, cyclophosphamide 2·5 mg/kg/day and cyclosporin 4 mg/kg/day, responded to anti-CD4 monoclonal antibody 25 mg/day for 7 days.[7] Two courses of treatment blocked disease progression for 12 months.

It appears that cyclosporin has much to offer for relapsing polychondritis and for some patients is the best choice. For others, cyclophosphamide has been more effective, and anti-CD4 antibodies look promising. The condition has a significant mortality and for the patient with adverse prognostic indicators[8] immunosuppressive drugs have an important place. There is insufficient evidence to show which of these agents is preferable.

Department of Dermatology,
Aberdeen Royal Infirmary,
Foresterhill,
Aberdeen AB9 2ZB, U.K.
**Beatson Institute for Cancer Research,*
Wolfson Laboratory for Molecular Pathology,
Bearsden, Glasgow, U.K.

A.D.Ormerod
L.J.Clark*

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Visual impairment secondary to rosacea

Sir, Rosacea is a chronic multiphasic vascular disorder of the skin. Its precise aetiology is unknown, but environmental or endogenous agents may initiate damage to connective tissue around upper dermal blood vessels,[1] resulting in unsupported and dilated leaky vessels. The process is manifest clinically by erythema, telangiectasia, papules, pustules, chronic facial oedema, and sebaceous gland hyperplasia.[2] Ocular involvement in rosacea is also felt to be vascular, or to have a vascular basis.[3] We describe a patient with unusual rosacea of the eyelids, which caused ptosis and significant impairment of vision.

An 80-year-old man presented with a 3-year history of progressive swelling and aching of his eyelids. The swelling on the right totally occluded his eye and he had intermittent complete ptosis on the left. Examination revealed an elderly man with marked kyphosis. There was gross bulbous swelling of the right eyelid causing a complete right ptosis. He also had left periorbital swelling, and rhinophyma (Fig. 1). Histological examination of a skin biopsy from the right upper eyelid showed loose degenerate collagen, a widespread chronic inflammatory infiltrate, and granuloma formation with necrosis. Computerized tomography of the orbits showed no evidence of an intra-ocular space occupying lesion.

Treatment with tetracycline and isotretinoin afforded no significant improvement, and a course of radiotherapy was unhelpful. A debulking procedure was performed on the right eyelid by a plastic surgeon. Grossly oedematous tissue was noted peroperatively. Excision of excess tissue resulted in good function of the right eyelid and restoration of vision.

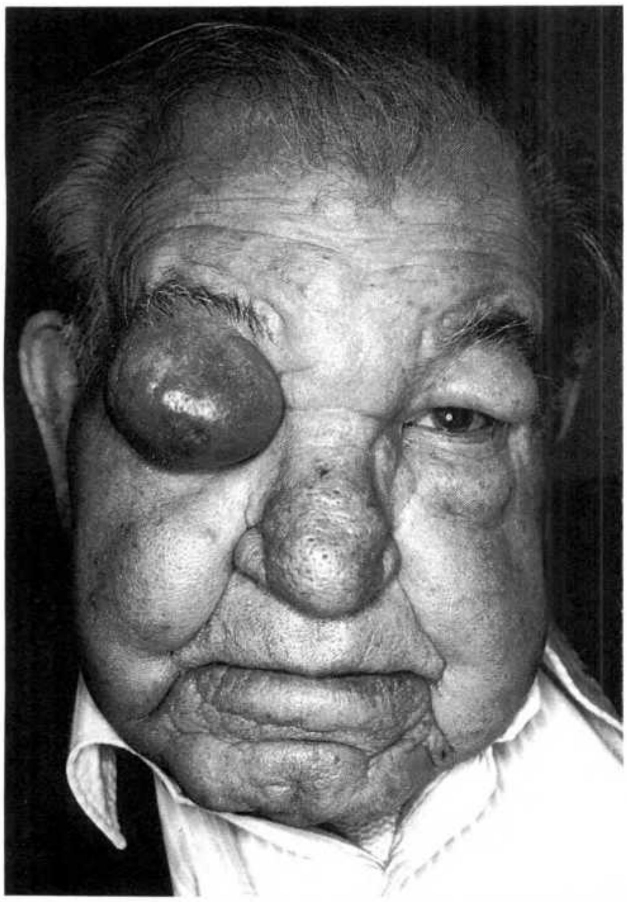

Figure 1. Blepharophyma occluding the right eye and causing left partial ptosis.

The prevalence of ocular rosacea in patients with cutaneous rosacea has been estimated to be 58% by an ophthalmologist.[3] Blepharitis is the commonest disorder and may result in recurrent chalazia and styes. In one series blepharitis affected 93% of patients with ocular rosacea,[4] and 86% suffered conjunctival hyperaemia (papillary conjunctivitis). Less common manifestations include meibomianitis, subnormal tear production, telangiectasia of the lid margin, superficial punctate keratopathy, iritis and episcleritis.[4] The ocular involvement is non-specific and ranges from mild disease to sight-threatening problems such as corneal neovascularization, thinning, ulceration, and perforation.[5]

Our patient demonstrated an unusual and florid variant of rosacea—blepharophyma, in addition to his rhinophyma. Florid facial 'phymas' may also be seen in the carcinoid syndrome.[6,7] Our patient's blepheral oedema may have been aggravated by his kyphosis. To our knowledge, rosaceous involvement of the eyelids sufficient to occlude vision has not previously been reported, and albeit very rare, may be added to the list of sight-threatening complications of rosacea.

The Skin Hospital,
35, George Road,
Edgbaston,
Birmingham B15 1BR

B.F.O'Donnell
I.S.Foulds

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British Journal of Dermatology (1992) **127**, 302.

Book Reviews

Biopsy Pathology of Melanocytic Disorders. W.J. Mooi and T. Krausz (1992). London: Chapman & Hall Medical. Pp. 433. Illustrations: 174 black and white 22 colour. ISBN 0-412-32350-8. Price £59.50.

This is the newest addition to a well-established series of surgical pathology texts ('surgical pathology' is what dermatologists call histopathology just as 'clinical appearances' are what pathologists call gross pathology). The series may not be familiar to dermatologists except for the recent general skin volume by Nigel Kirkham, but one or more volumes from it are to be found in most pathology laboratories.

The current volume concerns itself with melanocytic disorders and covers reactive, benign and malignant neoplastic lesions of the skin as well as having separate sections on prognostic factors, extracutaneous melanocytic lesions and a chapter on cytological diagnosis. This book appears at roughly the same time as the AFIP (Armed Forces Institute of Pathology) fascicle on '*Melanocytic Tumors of the Skin*' by Elder and Murphy, which is also a volume in a standard pathology series widely used by histopathologists, so that comparison is inevitable. The Biopsy Pathology (hardback) volume represents a Dutch/British collaboration, the AFIP (soft cover) volume is American; their prices are similar. The AFIP fascicle contains some colour clinical pictures, and the Biopsy Pathology book has some colour cytology illustrations. All routine histology is in black and white and cannot be faulted in either volume. The coverage is a little more extensive in the Biopsy Pathology volume. It contains descriptions of Cockade naevus and signet ring melanoma which are missing from the AFIP fascicle. Both books have extensive reviews of the 'dysplastic naevus' situation, and both are sensible and restrained, but the section in the Biopsy Pathology volume is probably the more balanced of the two.

I was a little disturbed to see a chapter on cytology of malignant melanoma, since aspiration of suspected melanoma seems to be a very bad idea. I was consequently relieved to read that the authors agree and feel that this technique should be limited to suspected secondary lesions when planning surgery.

The references are eclectic, are drawn widely from the world literature and show no obvious national bias.

The two chapters that might seem to be of little interest to the average dermatopathologist are those on extracutaneous melanocytic and extracutaneous melanotic lesions. In fact, if we consider diseases to be 'natural experiments', then these conditions are the 'natural controls' for such experiments. They are the situations that we have to study in order to see what is common to all melanomas and what is specific to the cutaneous lesions.

This book has no significant deficiencies and it does have some advantages over its closest rival. It has a good index, is well produced, well written and is good value for money. Every dermatologist and every pathologist who deals with melanomas needs this book. It is excellent.

D.Cotton

Topical Corticosteroids. Edited by H.I.Maibach and C.Surber (1992). Basel: Karger. Pp. 518. ISBN 3-8055-5332-3. Price £137.90.

Who is this book for? Those 'engaged in the development and use of topical corticosteroids' say the editors. Before you decide if you want to buy a copy for yourself, let me guide you through its 518 pages.

The first chapter reminds us that topical hydrocortisone came into clinical practice in 1952, and that other corticosteroids soon followed. Next we hear how their efficacy can be assessed in animals and then, in two chapters which overlap a great deal, in humans. Most of the tests on humans involving the suppression of inflammation (from croton oil, UVL, poison ivy, etc.) have not caught on, and the vasoconstriction test has ruled the roost since 1962. Stoughton's chapter on this test is well worth reading, and so is the one in which he uses it to show that tinkering with the ingredients of 'innovator formulations' can alter, often for the worse, the activity of cheaper 'generic copies'.

There follow straightforward chapters on the effects of changing the corticosteroid content of ointments, on percutaneous absorption, on thiosteroids, on clinical indications, and on side-effects. I have to admit that the 34 pages on 'structure-activity relationships using multivariate analysis techniques' went over my head.

Next, we delve into the use of topical corticosteroids in gynaecology, in paediatrics, in otology, and in the future: and still we have only reached page 235. Another 34 pages is taken up by a list of generic and brand names of corticosteroids in different countries. If you want to find out the differences between betnovate, betnovat, and betnevate, then look here.

The rest of the book (pp. 269–511) consists of many chapters, each of which is devoted to one of the main topical corticosteroids. These chapters are written by experts from the firms making the drugs and contain everything anyone would want to know, including the many trials in which one preparation has been pitted against another. A sense of déjà vu soon sets in, but it is good news that so many seem to be of 'superior efficacy' with 'favourable adverse-effect profiles'.

'To prudently generalize' (p. 165), I am sure that those who make topical corticosteroids will want a copy of this book: so too, perhaps, may the larger dermatology libraries. The book is well presented, although the writing varies from the brilliantly clear to the muddy and polysyllabic.

J.A.Savin

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News and Notices

The Second World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) Meeting, 8–11 September 1993, Los Angeles

The Second World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) meeting will be held in Los Angeles, California, U.S.A., on 8–11 September 1993. The scientific sessions will be devoted to the epidemiology, genetics, immunopathogenesis, and clinical aspects of sarcoidosis and other granulomatous disorders. Internationally acclaimed teachers, clinicians and scientists will help us bring the advanced basic science information to the bedside for diagnosing and managing complex problems related to granulomatous disorders. For further information please contact: Dr Om P. Sharma, Room 11-900, LAC/USC Medical Center, 1200 N. State Street, Los Angeles, CA, U.S.A. 90033. Telephone 01-213-226-7923. Fax 01-212-226-2738.

XIVth Course on Paediatric Dermatology, 13–17 April 1993, Arcachon, France

The XIVth Course on Paediatric Dermatology will be held on 13–17 April 1993 at Arcachon, France. The course is organized by Professors M.Larregue and J.Maleville. The programme will include Photodermatoses in Children, Melanocytic Tumours in Children, Oedematous Papules in Paediatric Dermatology, AIDS in Children, and clinical cases at the Bordeaux Children's Hospital. The course will be given in French. The number of participants is limited. The registration fee is FFr850. The deadline for registration is 28 February 1993. For further information please contact: Professor J.Maleville or Professor A.Taïeb, Service de Dermatologie, Hôpital Pellegrin-Enfants, Place Amélie Raba Léon, F. 33076 Bordeaux-Cedex, France. Telephone 56-79-56-22.

Preliminary announcement: International Symposium on Ozone–Sun–Cancer, Molecular and Cellular Mechanisms–Prevention, 17–20 May 1994, Paris, France

This International Symposium will be organized by L.Dubertret and R.Santus, General Secretary: P.Morlière, Institute for Skin Research at Hôpital Saint Louis and University Paris VII. The official language of the meeting will be English. For further information please contact: Dr P.Morlière, Laboratoire de Dermatologie, INSERM U.312, Hôpital Henri Mondor, 94010 Créteil, France. Telephone (33-1) 49-81-36-57. Fax (33-1) 49-81-35-16.

Introductory Course on the Biology of the Skin, Cambridge, December 1992

The annual introductory course 'The Biology of the Skin' will be held in Fitzwilliam College, Cambridge on 7–11 December 1992. This course is primarily for registrars and postgraduate students at an early stage of their training in dermatology but is open to other interested practitioners. Closing date for applications is 20 November, 1992. For further information please contact: the Conference Secretary (BoS), Postgraduate Medical Centre, Clinical School, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2SP, U.K. Telephone 0223 217105.

The RoC European Award in Photodermatology. An award of FFr50,000 (£5000)

This is an award which was created by RoC in 1990, and is granted to a national of the European Community Nations, in order to acknowledge a scientific work in the clinical aspects of photodermatology, whether published or not. The award will be made, for the third time, in 1993. The recipient of the RoC Award will be decided by a jury of scientists. Only work undertaken after 1 January 1991 will be considered for the award to be made in 1993. Submissions, written only in English or French, comprising a maximum of 10 typed pages, should be forwarded to: RoC S.A. Medical Department, 50 rue de Seine, 92703 Colombes Cedex, France, by 15 November 1992 at the latest. For further information, please contact: Anita Lee, Technical Manager, Laboratoires RoC (U.K.) Ltd, The Kirkgate, 19/31 Church Street, Epsom, Surrey KT17 4PF, U.K.

Joint Annual Meeting of the British Association of Dermatologists and the Canadian Dermatology Association, 6–10 July 1993, Oxford

The 73rd Annual Meeting of the British Association of Dermatologists will be a joint meeting with the Canadian Dermatology Association under the joint presidency of Dr William Gerstein (Canada) and Dr Terence Ryan (U.K.).

The meeting will be held in the Academic Centre at the John Radcliffe Hospital, Oxford from Tuesday 6 July to Saturday 10 July 1993.

Abstracts of papers and posters are invited for consideration by a Joint Abstract Selection Committee and those accepted as papers will be allotted 10 minutes for presentation plus time for discussion. Abstracts must be submitted on the official forms, which are avialable from: Miss Michelle Hardwicke, BAD Conference Office, 3 St Andrew's Place, London NW1 4LB, U.K.

The closing date for receipt of abstracts is Friday 15 January 1993 but applicants should note that the Scientific Committee will be administered by the Canadian Dermatology Association and extra time, therefore, should be allowed for postage to Canada.

Instead of the usual clinical/CPC session held on Saturday morning, there will be a 'Celebration of Dermatology' to be held in the Churchill Hospital, Oxford. Further details will be published in the autumn edition of the BAD Newsletter.

We were all saddened to hear of the deaths of Dr Robert P. Warin and his wife Anne in July. A memorial service is to be held at 12 noon on Friday 23 October 1992 at St Mary Redcliffe Church, Bristol.

British Journal of Dermatology (1992) 127, 305–311.

Distribution of interleukin 1 receptor antagonist protein (IRAP), interleukin 1 receptor, and interleukin 1α in normal and psoriatic skin. Decreased expression of IRAP in psoriatic lesional epidermis

M.KRISTENSEN,* B.DELEURAN,† D.J.EEDY,‡ M.FELDMANN, S.M.BREATHNACH‡ AND F.M.BRENNAN

Charing Cross Sunley Research Centre, Hammersmith, London, U.K
**Department of Dermatology, University of Aarhus, Denmark*
†Kennedy Institute of Rheumatology, Hammersmith, London, U.K
‡St John's Institute of Dermatology, St Thomas's Hospital, London, U.K.

Accepted for publication 6 May 1992

Summary The distribution of interleukin 1α (IL-1α), type 1 interleukin 1 receptor (IL-1R), and the interleukin 1 receptor antagonist protein (IRAP), was investigated in biopsies of normal skin, and in uninvolved and lesional skin of patients with psoriasis, using specific monoclonal antibodies. We report the novel finding that IRAP is distributed throughout the living layers of the epidermis in normal skin, and is also associated with sebaceous glands and eccrine sweat glands. Our finding that the inhibitor protein IRAP is present in areas where the pro-inflammatory cytokine IL-1α is distributed provides strong evidence in favour of a cytokine regulatory system in normal skin. We further document for the first time that IL-1R in normal skin is localized to the living layers of the epidermis, sebaceous and eccrine glands, as well as to a prominent network of dermal dendritic cells, and upper dermal blood vessels. There was a consistent reduction in the level of IRAP expression in lesional compared with uninvolved skin in biopsies from six out of seven psoriasis patients. Decreased IRAP expression in lesions of psoriasis indicates that alterations in the level of this inhibitor protein may be important in the pathogenesis of inflammatory skin conditions.

The presence within normal human epidermis of material with the bioactivity of interleukin 1 (IL-1), which cross-reacts immunochemically with both the IL-1α and IL-1β forms, is now well established.[1–11] The reported localization of intraepidermal IL-1 as determined by immunostaining has varied between different studies, probably as a result of technical factors, but a recent study involving a range of fixatives and a panel of antibodies has revealed that keratinocytes at various stages of maturation display both membrane-associated and cytosolic IL-1α and β immunoreactivity.[10] In addition, both IL-1α and β have been detected in the clear, but not the dark, cells of the secretory coil of eccrine sweat glands in the dermis, as well as in the luminal and basal cells of the dermal coiled and straight ducts, and in the upper intradermal eccrine ducts.[9] IL-1α appears to be the major IL-1 species in terms of both immunohistochemical staining[8] and of the amount of bioactive material recovered from the epidermis[11–14] and from eccrine sweat,[15] although IL-1β immunoreactivity without corresponding IL-1β bioactivity has been identified in extracts of normal human heel stratum corneum.[13] The steady-state rate of epidermal IL-1α mRNA synthesis appears very low, and the large amounts of epidermal IL-1α may therefore be the result of storage.[14] Although *in vivo* ultraviolet light (UVB) irradiation results in local and systemic immunosuppression,[16] the expression of IL-1 mRNA and protein by keratinocytes is enhanced by UVB irradiation.[6,17,18] It is therefore of interest that UVB-irradiated murine keratinocytes were found to produce a specific inhibitor of IL-1 *in vitro*, termed epidermal cell-derived contra IL-1 (EC contra IL-1) with a molecular weight of approximately 40 kDa.[19] This suggests that mechanisms exist for the regulation of IL-1 bioactivity in normal epidermis. The nature of EC contra IL-1 is currently unknown.

It has been proposed that keratinocyte production of

Correspondence: Dr S.M.Breathnach, St John's Institute of Dermatology, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, U.K.

IL-1, in conjunction with that of other epidermal cytokines, may play a central role in the immunopathogenesis of skin inflammation.[20] Recombinant IL-1 is pro-inflammatory *in vivo*;[21] IL-1 enhances epidermal Langerhans cell-dependent T-cell activation in the skin,[22] and with tumour necrosis factor (TNF-α), induces production of mRNA for the chemotactic cytokine interleukin 8 (IL-8).[23] In addition, IL-1, with other keratinocyte-derived cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL-6) and transforming growth factor α (TGF-α), stimulates keratinocyte proliferation.[24,25] For these reasons, interest has been focused on the potential role of IL-1 and other cytokines in the pathogenesis of psoriasis. Increased levels of TGF-α[26,27] and of IL-6[28,29] have been documented in psoriasis; immunoreactive IL-8 has been reported as increased in some[30] but not other[31] studies. There has been a single report of increased IL-1α expression in psoriatic epidermis,[8] whereas the majority of studies have described a reduction in IL-1 bioactivity,[32,33] although an increase in immunoreactive, but non-functional IL-1β was observed.[34]

Recent work from several laboratories has led to the isolation and cloning of an IL-1 receptor binding protein which has IL-1 inhibitory activity.[35–37] This recombinant molecule, termed IL-1 receptor antagonist protein (IRAP), has a molecular mass of 18–23 kDa and shows significant homology to IL-1α and IL-1β. An IL-1 antagonist protein equivalent to IRAP has been shown to be produced by monocytes in response to differentiation signals such as adherent IgG,[35] GM-CSF[38] and transforming growth factor β (TGF-β).[39] We speculated that the IL-1 inhibitory activity previously described in normal and psoriatic epidermis might be due, at least in part, to the presence of IRAP. IRAP has in fact very recently been reported to be produced by cultured keratinocytes.[40] It has been postulated that, although IL-1 activity is not increased in psoriasis, levels of the IL-1 receptor might be increased.[41] Therefore, we investigated the presence and distribution of IL-1α, its receptor, and the naturally occurring antagonist IRAP in normal epidermis, and compared their distribution and intensity between uninvolved and involved lesional epidermis obtained from psoriasis patients, using specific monoclonal antibodies.

Methods

Reagents

Human recombinant IRAP protein (rIRAP), and a mixture of mouse monoclonal antibodies directed against IRAP (I-4, I-10, I-12, I-15), were kindly provided by Dr Ann Berger, Upjohn Co., Kalamazoo, MI, U.S.A. The specificity of the individual antibodies for IRAP was confirmed as follows (A.Berger, personal communication and unpublished data). Antibodies I-4, I-10, and I-15 neutralized IRAP bioactivity; binding of these antibodies to IRAP coated on ELISA plate wells was competitively inhibited by their pre-incubation with intact soluble IRAP, but not by fragments of IRAP generated by proteolytic cleavage, suggesting that they are directed against conformationally determined epitopes. Antibody I-12 failed to neutralize the bioactivity of IRAP; its immunoreactivity was blocked by a very large C-terminal cleavage fragment of IRAP composed of amino acids 97–146, but not by rIRAP, suggesting that it reacts preferentially with denatured IRAP. Human recombinant IL-1α was a gift from Hoffman La Roche (Nutley, New Jersey, U.S.A.) and monoclonal anti-IL-1α was a gift from M.Okazaki of Dainippon Pharmaceuticals, Japan. Monoclonal anti-IL-1 receptor was a gift from Dr Steven Dower (Immunex). Normal mouse IgG1 (Sigma, Poole, Dorset, U.K.) served as a control for immunostaining. Polyclonal goat anti-mouse IgG and APAAP (alkaline phosphatase, anti-alkaline phosphatase) complex were obtained from Dako, Copenhagen, Denmark.

Skin biopsies

Samples of normal perilesional skin ($n=10$) were obtained from subjects undergoing minor dermatological surgery procedures. In addition, paired biopsies of involved and adjacent uninvolved areas were obtained from individual patients ($n=7$) with stable plaque psoriasis. Psoriasis patients were not taking systemic therapy, and had not used topical therapy at the biopsy sites for at least 7 days. Informed consent was obtained from all patients. Skin biopsies were snap-frozen, and stored at −70°C until required.

Immunohistological techniques

Six-micrometre cryostat sections from each biopsy were fixed in methanol/acetone (1:1) for 10 min at −20°C, washed in 0·05 mM Tris-buffered saline (TBS) for 2 min at 0°C, then blocked for 20 min with 20% normal goat serum (NGS) (Sigma). Sections were incubated with the primary antibody (monoclonal anti-IRAP 20 mg/ml, anti-IL-1α 6 mg/ml or anti-IL-1R 10 mg/ml) diluted 2% v/v in NGS/TBS overnight at 4°C. Mouse monoclonal antibodies were detected with goat anti-mouse IgG

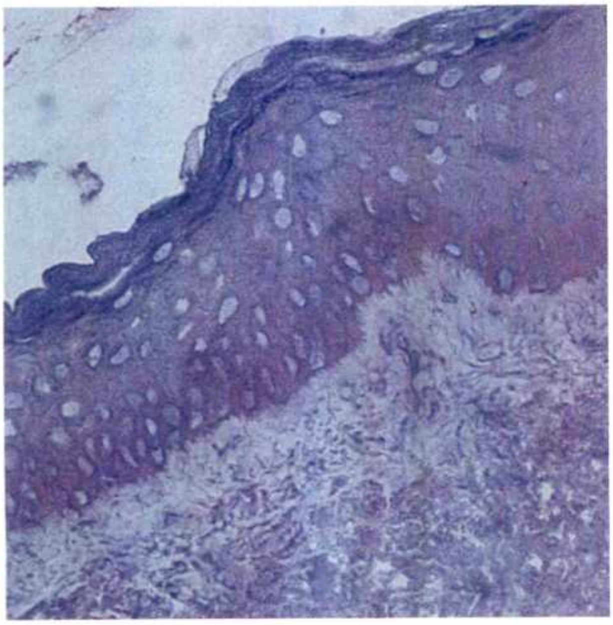

Figure 1. Distribution of immunoreative IL-1α in normal human skin. IL-1α is localized predominantly to the basal layers of the epidermis (× 250).

(Dako, Copenhagen, Denmark) diluted 1 : 30 in 2% NGS/TBS for 30 min at room temperature, then incubated with the APAAP complex (Dako) diluted 1:40 in 2% NGS/TBS for 30 min. To enhance the signal the goat anti-mouse IgG and APAAP step was repeated. The sections were then incubated with fast red (Sigma) and colour-developed after 10 min. The reaction was stopped with distilled water. Tissues sections were all counterstained with haematoxylin. Positive staining was visualized by bright pink coloration. Uninvolved and lesional psoriatic skin from the same patient was processed and stained simultaneously to exclude inter-experimental variation.

Specificity controls

To confirm specificity of the monoclonal antibodies for their respective ligands, the antibodies were tested against a variety of ligands (rIRAP, IL-1α, IL-1β and TNF-α) in an enzyme-linked immunoassay. Monoclonal anti-IL-1α antibody bound IL-1α in a dose-dependent manner, but not rIRAP, IL-1β, or TNF-α. Similarly

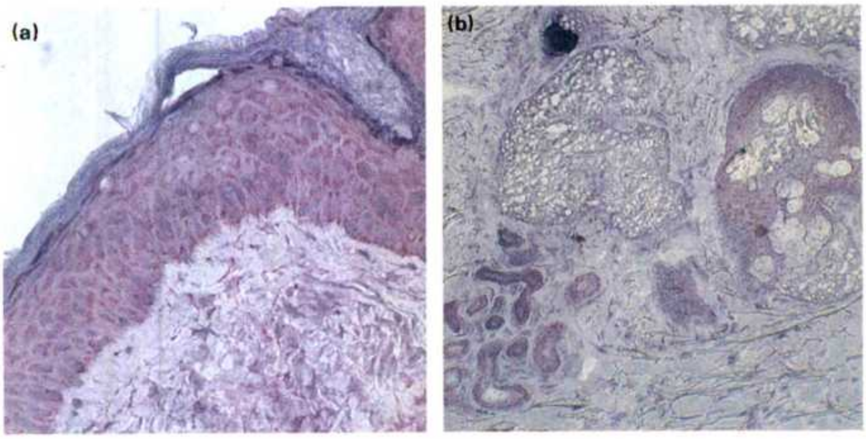

Figure 2. (a) Immunoreactive IL-1R is distributed throughout the living layers of the epidermis in normal human skin. In addition, scattered upper dermal dendritic cells stain positively (× 250). (b) Immunoreactive IL-1R in dermal eccrine and sebaceous glands (× 250).

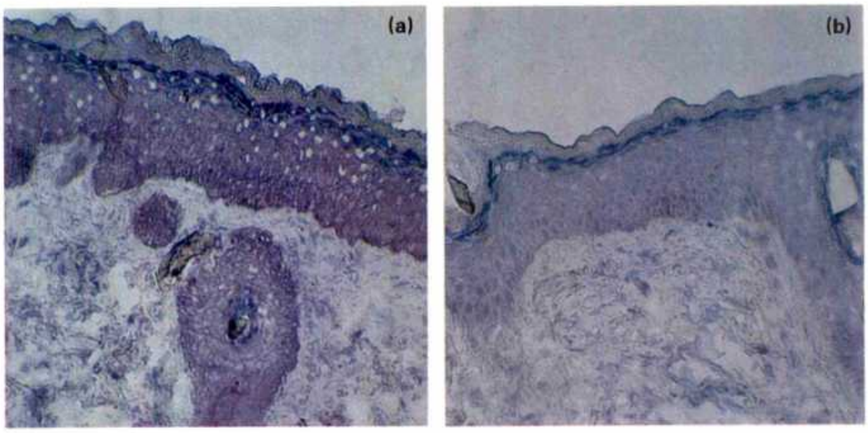

Figure 3. Distribution of immunoreactive IRAP in human skin (a) IRAP is present throughout the living layers of the epidermis in normal human skin. (b) Immunostaining is eliminated by pre-incubation of anti-IRAP antibodies with 10 mg/ml recombinant IRAP (× 125).

monoclonal anti-IRAP antibodies only bound recombinant IRAP in the ELISA (data not shown). Monoclonal anti-IL-1 receptor antibody showed no binding to either rIRAP, IL-1α or IL-1β. The binding of anti-IL-1 receptor is specific and is blocked by ligand (S.Dower, personal communication).

To confirm the specificity of the staining the diluted antibody was pre-incubated overnight at 4°C, either alone or in the presence of 10 mg/ml of the relevant ligand, i.e. monoclonal anti-IRAP antibody with recombinant IRAP, monoclonal anti-IL-1α with rIL-1α, and monoclonal anti-IL-1 receptor with rIL-1α (see above). It was possible to totally block the staining for IL-1α and IRAP, whilst the staining for IL-1 receptor was strongly inhibited. Mouse IgG1 (same isotype as monoclonals) was included as a specificity control.

Microscopy and analysis of staining

Immunohistochemical staining with antibodies to IL-1α, IL-1R, and IRAP was assessed under the light microscope independently by three observers (M.K., D.E.J., S.M.B), and a consensus view was obtained. For the normal skin samples, the distribution of staining in the epidermis and in association with dermal structures, including blood vessels and appendages such as sebaceous glands and eccrine sweat glands, was recorded. Similarly, comparison was made between the distribution and intensity of staining with the various antibodies between the paired samples of involved and uninvolved skin from psoriasis patients. It was not possible to carry out this in a blinded fashion, because of the distinctive histological features of lesional psoriasis skin. The intensity of staining in the epidermis was graded according to the following system: 0, absent; 1, weak; 2, moderate; 3, strong.

Results

Distribution of IL-1α, IL-1R, and IRAP in normal human skin

IL-1α, IL-1R, and IRAP distribution in normal skin was detected by immunostaining using specific monoclonal antibodies, and found to be specific by inhibition of monoclonal antibody staining by pre-incubation with relevant ligand (see Methods). Mouse IgG1 antibody which served as a control for non-specific binding was negative on all skin sections.

Staining with anti-IL-1α antibody was predominantly in the basal layers of the epidermis in seven of the 10 biopsies (Fig. 1). In the remaining three biopsies staining was distributed throughout the epidermis with the exception of the stratum corneum. When present in the sections, the secretory coils of eccrine sweat glands, the sebaceous gland lobules, and upper dermal blood vessels were also stained (not shown).

In contrast with anti-IL-1α, epidermal staining with anti-IL-1R antibody was detectable within both the basal and upper layers of the epidermis in nine of the 10 biopsies (Fig. 2a). Scattered upper dermal dendritic cells also stained positively with anti-IL-1R in all biopsies (Fig. 2a). IL-1R staining was detected in eccrine sweat glands and sebaceous glands (Fig. 2b), as described above for IL-1α. Staining with anti-IL-1R was also found in association with upper dermal blood vessels, and with arrector pili muscle fibres (not shown).

Table 1. Intensity[*] of epidermal staining in lesional skin compared with uninvolved skin from patients with psoriasis

	Anti-IL-1α		Anti-IL-1 receptor		Anti-IRAP	
Patient	U[†]	L[‡]	U	L	U	l
1						
Basal	2	3	3	3	2	2
Upper	1	3	3	3	2	3
2						
Basal	3	1	3	2	3	1
Upper	2	1	3	2	3	1
3						
Basal	2	3	3	2	2	1
Upper	2	3	3	2	2	2
4						
Basal	2	1	2	2	3	1
Upper	2	1	3	2	3	2
5						
Basal	2	3	3	2	3	1
Upper	2	3	3	2	2	1
6						
Basal	2	1	2	2	3	2
Upper	2	1	2	3	3	2
7						
Basal	3	2	2	2	2	1
Upper	2	2	2	2	2	1
MEAN[§]						
Basal	2·3 ±0·5	2·0 ±1·0	2·6 ±0·5	2·1 ±0·4	2·6 ±0·5	1·3 ±0 5[**]
Upper	1·9 ±0·4	2·0 ±1·0	2 7 ±0·5	2 3 ±0 5	2·4 ±0·5	1·7 ±0·8

[*] Intensity of staining was graded as follows: 0, absent; 1, weak; 2, moderate; 3, strong.

[†] U, uninvolved skin; [‡] L, lesional skin; [§] mean score staining intensity.

[**] $P<0{\cdot}004$ between lesional and uninvolved skin.

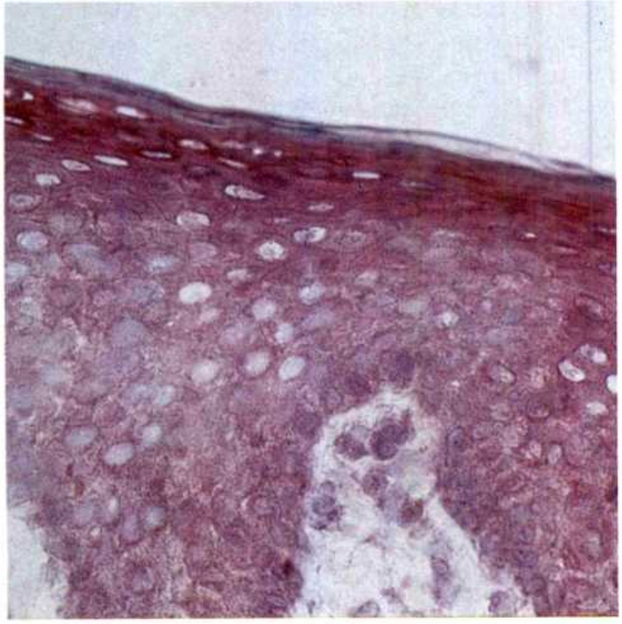

Figure 4. IL-1R immunoreactivity is distributed throughout the epidermis, including the parakeratotic stratum corneum, in psoriatic skin (× 250).

Of particular interest was the observation that antibodies against the IL-1 receptor antagonist protein IRAP also stained the epidermis in a distribution similar to that detected with anti-IL-1R antibody (Fig. 3a). This immunostaining was inhibited by pre-incubation of the monoclonal antibody mixture with ligand, namely rIRAP (Fig. 3b). Eccrine ducts, sebaceous glands, and an area of arrector pili muscle were also stained with anti-IRAP (not shown).

Distribution of immunoreactive IL-1α, IL-1R, and IRAP in psoriasis skin

The overall distribution of staining in both uninvolved and lesional skin from psoriasis was broadly qualitatively the same as that observed in normal human skin, for the antibodies directed against IL-1α, IL-1R and IRAP. However, in psoriatic skin, staining with anti-IL-1α was seen throughout the living layers of the epidermis in all specimens (Table 1), rather than being predominantly basal; there was prominent staining of tortuous papillary blood vessels with this antibody. In addition, in the great majority of specimens, staining with all three antibodies was detectable in the parakeratotic stratum corneum in lesional psoriatic skin, in contrast with the absent staining in uninvolved skin. Figure 4 illustrates IL-1R staining in this region. With antibodies to IL-1α and IL-1R, there was no consistent alteration in the intensity of staining of the basal and upper layers of the epidermis (Table 1). In contrast, in six of the seven lesional psoriasis skin biopsies, the intensity of basal and upper epidermal staining with anti-IRAP was consistently reduced compared with uninvolved psoriatic skin (Table 1, Fig. 5a and b). The reduction in basal epidermal IRAP immunoreactivity in lesional compared with uninvolved psoriasis skin reached statistical significance (Student's *t*-test: $P < 0{\cdot}004$).

Discussion

We report here the novel finding that, in normal skin, IRAP is distributed through the living layers of the epidermis, within the lobules of sebaceous glands, and in association with eccrine sweat glands. We also

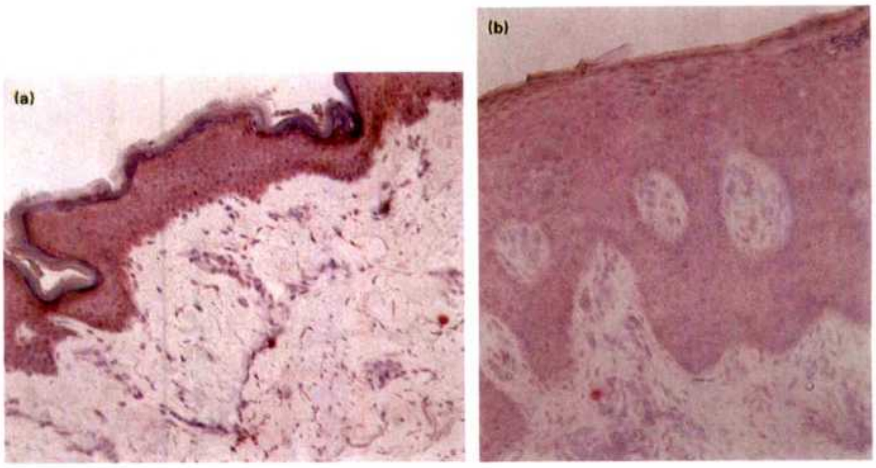

Figure 5. (a) Marked staining with anti-IRAP of all the living layers of the epidermis in uninvolved psoriatic skin. (b) Decreased staining intensity with anti-IRAP of acanthotic epidermis in involved psoriasis skin (note identical magnification, × 100).

document for the first time that IL-1R in normal skin is localized to the living layers of the epidermis, sebaceous and eccrine glands, as well as to a prominent network of dermal dendritic cells, and upper dermal blood vessels. The nature of the IL-1R-positive dermal dendritic cells is currently under investigation. We confirm previous work showing that IL-1α is present in human epidermis[1–11] and in eccrine sweat glands,[9] and comment on its distribution in association with sebaceous glands and upper dermal blood vessels. Our finding that the inhibitor protein IRAP is present in areas in which the proinflammatory cytokine IL-1α and its receptor is found, provides strong evidence in favour of a cytokine regulatory system in the skin.

It might at a first glance be difficult to envisage a significant role for IRAP, given that it is so readily possible to extract large amounts of bioactive IL-1α from normal human skin. However, IL-1α is stored as an inactive membrane-bound precursor, which needs to be proteolytically digested to the active form. An intracellular form of IRAP has recently been characterized in cultured epithelial cells including keratinocytes. This intracellular form of IRAP lacks a leader sequence, and it has been proposed that it may function as an inhibitor of intracellular IL-1.[42]

We found that, in contrast with normal skin, in psoriatic skin IL-1α was not predominantly restricted to the basal layers of the epidermis, but was present throughout the spinous layer and within the parakeratotic stratum corneum; prominent staining of tortuous papillary blood vessels was noted, particularly in lesional skin. However, there was considerable inter-patient variation in the intensity of staining with anti-IL-1α, and no consistent difference in the degree of expression of IL-1α between uninvolved and lesional skin of psoriasis patients could be determined. Similarly there was no consistent difference in upper and basal epidermal staining for IL-1R between uninvolved and psoriasis skin, although like IL-1α the parakeratotic stratum corneum stained positively for IL-1R. In contrast with IL-1α and IL-1R immunoreactivity, there was a consistent trend towards reduced expression of IRAP in the basal and upper layers of lesional skin, compared with uninvolved skin, in six out of seven patients with psoriasis. The biological significance of this finding is at present difficult to interpret, in view of the documented reduction in bioactive IL-1α in psoriatic skin.[32,33] IL-8 immunoreactivity has been demonstrated in both normal and psoriatic skin. In one study it was reported to be absent in highly active psoriasis, and focally reduced in plaque-type psoriasis,[31] and it was suggested that this indicated release of IL-8 from a preformed pool in active lesions. By analogy, one interpretation of our observation that IRAP is consistently reduced in psoriasis lesions would be that this inhibitor of IL-1 is released in lesional skin. Alternatively, reduced expression of IRAP in lesional psoriasis might reflect decreased synthesis, and consequent intracellular storage, of IRAP. Studies of the rate of synthesis of IRAP mRNA in normal and psoriatic skin will be necessary in order to further address this conundrum.

Acknowledgments

This work was supported by the Christian & Ottilia Brorsons Travel Foundation, A.C. Bangs Foundation, Institute of Experimental & Clinical Research, University of Aarhus, Denmark, the Arthritis & Rheumatism Council, and the Coisy Bequest.

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British Journal of Dermatology (1992) 127, 312–317.

Intracellular expression of type VII collagen during wound healing in severe recessive dystrophic epidermolysis bullosa and normal human skin

J.A.McGRATH, I.M.LEIGH* AND R.A.J.EADY,
St John's Institute of Dermatology, UMDS (St Thomas's campus), St Thomas's Hospital, London, U.K.
**Department of Experimental Dermatology, The Royal London Hospital, London, U.K.*

Accepted for publication 13 May 1992

Summary The ability of keratinocytes to synthesize basement membrane components *in vivo* during wound healing in normal human skin and in severe recessive dystrophic epidermolysis bullosa (RDEB) was investigated. Indirect immunofluorescence using anti-type VII collagen (VIIc, recognizing the globular non-helical component of the molecule), anti-type IV collagen, anti-laminin and bullous pemphigoid antisera, was performed on biopsies of intact skin and of healing skin taken between 7 and 14 days after dermatome injury (upper to mid-dermal wounding) in eight patients with severe RDEB and in seven normal subjects. Baseline anti-type VIIc immunofluorescence showed completely absent staining of the epidermis, dermis and dermo-epidermal junction in severe RDEB samples, and bright linear dermo-epidermal junction fluorescence in normal human skin. In 5/5 normal human skin samples taken 9–12 days post-wounding, some type VIIc expression was noted within basal cells as well as in a continuous or interrupted linear distribution at the basement membrane zone. In all the severe RDEB biopsies sampled between days 10 and 13 (5/5), anti-VIIc fluorescence was also seen with varying intensity within basal and lowermost suprabasal cells, and in one day 14 sample at the dermo-epidermal junction. Low levels of intracellular type IVc were seen in both groups, but only in those samples taken 7–9 days after injury; later biopsies showed only continuous dermo-epidermal junction staining. Linear basement membrane zone labelling with laminin and bullous pemphigoid antisera was seen in all samples in both sets of subjects, even at day 7, but there was no detectable intracellular antisera staining. The finding of anti-type VII collagen immunofluorescence staining within the lower epidermis in severe RDEB during wound healing implies that these keratinocytes, under certain circumstances, have an inherent capacity to synthesize type VIIc, or at least part of the non-collagenous globular region of the molecule, and that a complete absence of type VIIc is not the primary abnormality in the most severe form of RDEB.

Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited mechano-bullous disorder characterized clinically by fragile skin and mucous membranes, blisters, erosions, and chronic scarring. The most severe form of the condition is typified morphologically by an absence of recognizable anchoring fibrils at the dermo-epidermal junction and blister formation below the lamina densa.[1–3] Anchoring fibrils are mainly composed of type VII collagen (VIIc),[4] and an absence of immunofluorescence staining with anti-type VIIc antibodies has been widely used in the diagnosis of severe RDEB.[5–7] It is not clear, however, whether the abnormality of type VIIc in severe RDEB represents complete absence, altered synthesis, abnormal polymerization or excessive breakdown of the type VIIc molecule. In normal human skin, type VIIc is synthesized by both keratinocytes and fibroblasts,[8,9] although the slow turnover of basement membrane components in intact skin is such that detection of type VIIc by immunofluorescent anti-type VIIc labelling is usually limited to linear staining at the dermo-epidermal junction. Studies of single biopsies of normal or EB skin are only able to give limited information on the dynamics of type VIIc synthesis. However, a process such as wound healing, associated with an increased synthesis of basement membrane components, may provide a useful model for studying the synthesis and transport of type VIIc and other macro-

Correspondence: Professor R.A.J.Eady, St John's Institute of Dermatology, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, U.K.

molecules of the basement membrane, especially if analysis of sequential biopsies is possible. Such an approach might enable one of the potential abnormalities of type VIIc in severe RDEB to be investigated.

Methods

Subjects

Eight patients (five male, three female; age range 18–44 years, mean age 27·9 years) with severe RDEB were studied. The diagnosis was based both on characteristic clinical features of severe, generalized, mutilating skin blistering and scarring, and on skin biopsy evidence of a sublamina densa level of blister formation and unrecognizable anchoring fibrils on electron microscopy. Each patient was undergoing plastic surgery to release chronic scarring-associated hand contractures and having a split-thickness skin graft taken from the anterior aspect of the thigh with a lubricated dermatome. Seven normal control subjects (six males, one female; age range 28–68 years, mean age 48·9 years) having a similar split-thickness skin graft procedure (e.g. for tattoo removal), using the thigh as donor site, were also studied.

Table 1. Number of sequential skin samples from patients with recessive dystrophic epidermolysis bullosa (RDEB) and normal controls (NHS) after wounding

	Time of biopsies (day after wounding)								
	0	7	8	9	10	11	12	13	14
RDEB	8	1	—	1	2	—	2	1	1
NHS	7	1	—	1	3	—	1	—	1

Skin samples

Skin specimens for baseline assessment (day 0) were taken from the edge of the split-thickness skin graft specimen immediately after dermatome removal from the thigh. Following informed consent, each subject then had one further biopsy (6-mm punch biopsy in controls, shave biopsy in severe RDEB group) taken from the healing donor site on the thigh 7–14 days later (see Table 1 for timing of biopsies).

Immunofluorescence

Immediately after collection, skin samples were embedded in OCT compound (Miles laboratories, Elkhart, U.S.A.) and snap-frozen in liquid nitrogen. Cryostat sections (5 μm) were then cut, air-dried, pre-incubated with either normal rabbit or normal goat serum at room temperature for 15 min to reduce non-specific secondary antibody binding, and then incubated in primary antibody for 1 h at 37°C (Table 2). After washing twice in phosphate-buffered saline (PBS) for 30 min, the sections were incubated in fluorescein-conjugated anti-IgG for 1 h at 37°C, washed in PBS and distilled water, and mounted in glycerol/PBS containing 0·1% *p*-phenylenediamine. Sections incubated in negative control primary sera (rabbit, goat or normal human serum) followed by fluorescent secondary antibody, were also examined.

Results

Normal human skin

Day 0. Baseline immunofluorescence showed continuous linear dermo-epidermal junction (DEJ) labelling with anti-VIIc (Fig. 1a), anti-IVc (Fig. 1b), anti-laminin, and bullous pemphigoid (Fig. 1c) antisera. Fluorescent staining around blood vessels, nerves and epidermal

Table 2. Antibodies used in immunofluorescence study

Primary antibody	Comments	Dilution	Secondary antibody	Dilution
LH 7:2	Mouse monoclonal (globular region of type VII collagen)	1·2	Rabbit anti-mouse*	1:50
Anti-type IV collagen*	Rabbit polyclonal	1:100	Goat anti-rabbit*	1:50
Anti-laminin*	Rabbit polyclonal	1:100	Goat anti-rabbit*	1:50
Bullous pemphigoid antisera†	Human polyclonal	1:10	Goat anti-human*	1:50

*ICN Immunobiologicals, U.K.

†Recognizes 230-kDa protein by immunoblotting.

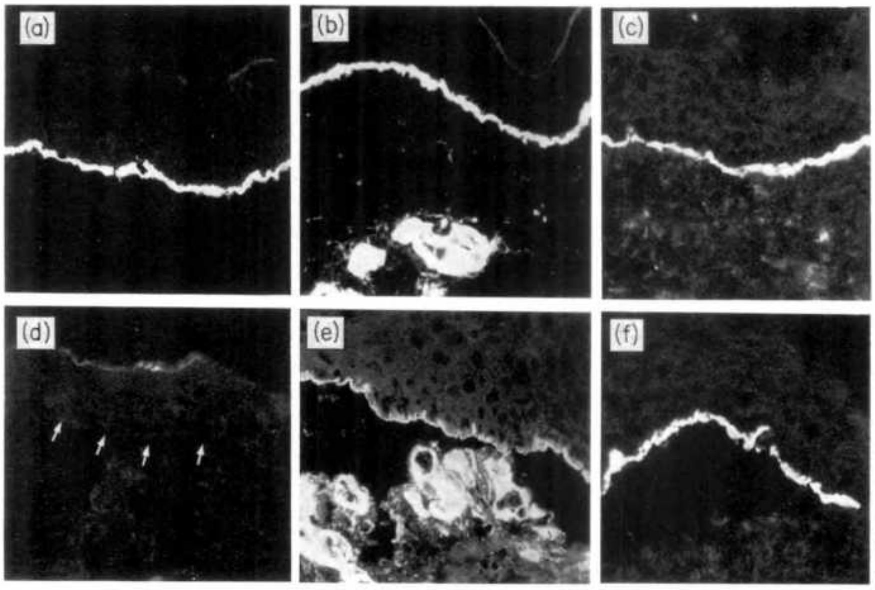

Figure 1. Baseline immunofluorescence findings in normal human skin (NHS, top row) and severe recessive dystrophic epidermolysis bullosa (RDEB, bottom row). (a, d) Anti-type VII collagen labelling. There is linear staining along the dermo-epidermal junction (DEJ) in NHS (a), but completely negative labelling of the DEJ (arrowed) in severe RDEB (d). (b, e) Anti-type IV collagen labelling. Both groups have continuous fluorescence along the DEJ and around dermal vessels and nerves. In RDEB (e), the antibody stains the blister roof. (c, f) Bullous pemphigoid antiserum labelling. Both sets of patients have linear staining of the DEJ, which localizes to the blister roof in RDEB (f). (Magnifications: a ×95, b ×65, c ×100, d ×65, e ×135, f ×100).

appendages was also seen with type IVc and laminin antisera. No intraepidermal labelling was seen.

Day 7–14. Anti-VIIc labelling was not noted before day 9. Thereafter it was seen in a continuous or interrupted linear pattern along the DEJ as well as in granular intracellular deposits within basal keratinocytes (Fig. 2a), although by day 14 no intraepidermal staining was detected and only continuous linear DEJ fluorescence was found. In those sections with continuous DEJ staining, the labelling was most intense around hair follicles, when compared with adjacent interfollicular epidermis (Fig. 2c). Some intraepidermal labelling with anti-type IVc antiserum was seen on the day 7 sections (Fig. 2f) in addition to interrupted linear DEJ staining and pronounced upper dermal fluorescence in association with new blood vessel formation. By day 10 both the epidermis and DEJ had similar appearances to the baseline state. Laminin and bullous pemphigoid antisera fluorescence was linear and continuous at the dermo-epidermal junction in all wound healing specimens examined, with no intracellular labelling seen at any stage. None of the control samples with fluorescent secondary antibody alone had any intraepidermal, DEJ, or dermal staining. There was some slight variability in the intensity of the labelling within samples from different individuals taken at similar times after injury. This might be explained by differences in the original depth of wounding by the dermatome giving rise to variations in the pattern or rate of keratinocyte migration and differentiation. Histologically, there was considerable epidermal hyperplasia at the time of intraepidermal collagen labelling during wound healing.

Severe recessive dystrophic epidermolysis bullosa

Day 0. Baseline anti-VIIc labelling studies showed a complete absence of staining in all eight severe RDEB subjects (Fig. 1d). Type IVc (Fig. 1e), laminin, and bullous pemphigoid (Fig. 1f) antisera had a similar labelling pattern to normal human skin, although in areas of blistering or dermo-epidermal separation all antibodies stained the roof of the specimen.

Day 7–14. At day 7, no anti-VIIc labelling was noted (as in normal human skin). However, in all five biopsy samples taken 10–13 days after wounding some anti-VIIc fluorescence within the basal keratinocytes and lowermost suprabasal cells (Fig. 2b) was seen, albeit less bright than in normal human skin. In the single day 14 biopsy, faint, discontinuous anti-VIIc fluorescence was present at the DEJ (Fig. 2d). Slightly increased dermal fluorescence, possibly in association with fibroblasts, was also found in all the 10–14 day biopsies. Type IVc (Fig. 2g and 2h), laminin and bullous pemphigoid (Fig. 2e) antisera labelling showed similar characteristics to the normal controls.

Discussion

The LH 7:2 monoclonal antibody[10] recognizes an epitope on the non-collagenous globular region of type

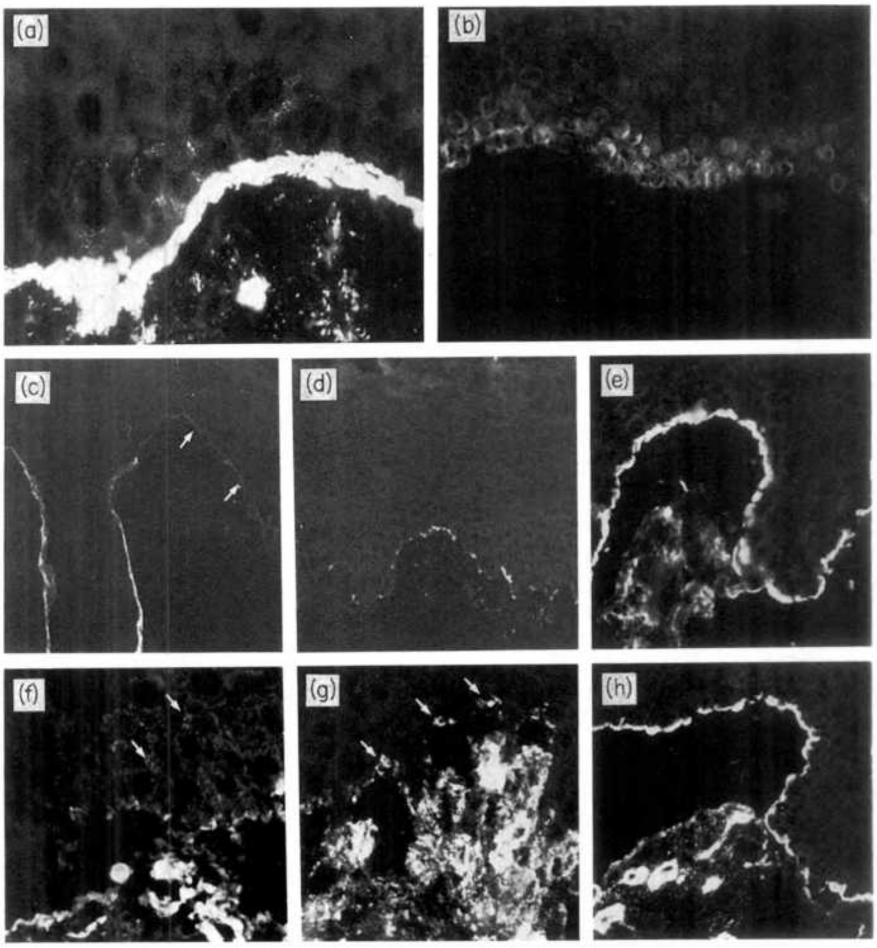

Figure 2. (a) Anti-type VII collagen NHS day 10. Discrete granular staining is seen within basal and lowermost suprabasal cells in addition to continuous linear DEJ fluorescence (× 260). (b) Anti-type VII collagen RDEB day 12. Intracellular fluorescence is present within basal and some suprabasal cells (cf. day 0 appearances in Fig. 1d). No definite DEJ staining is seen (× 140). (c) Anti-type VII collagen NHS day 12. There is continuous linear staining of the DEJ, but no intraepidermal fluorescence. The follicular epithelium has brighter labelling than the interfollicular epidermis (arrowed, × 45). (d) Anti-type VII collagen RDEB day 14. No keratinocyte labelling is seen, but scanty, discontinuous fluorescence is noted beneath the hyperplastic epidermis at the DEJ (cf. day 0 appearances in Fig. 1d, × 90). (e) Bullous pemphigoid antiserum RDEB day 10. There is continuous linear DEJ fluorescence which localizes to the roof of blistered skin (× 155). (f) Anti-type IV collagen NHS day 7. Granular fluorescence is seen intracellularly within the lower epidermis (arrows). Some dermo-epidermal separation is noted even in NHS at this stage in wound healing (× 150). (g) Anti-type IV collagen RDEB day 7. Some discontinuous labelling (arrows) is observed both at the DEJ and within some basal cells. Some dermo-epidermal separation/early blister formation is also present (× 135). (h) Anti-type IV collagen RDEB day 14. Near-continuous linear DEJ fluorescence is seen, with the antibody staining the roof of separated skin (× 140).

VIIc, a major component of anchoring fibrils.[4,5,11] It has been widely used in the diagnosis and classification of dystrophic EB.[6,12] In nearly all cases of severe, mutilating, generalized RDEB there is absent immunofluorescence staining of the dermo-epidermal junction with this antibody, as found in the present study. However, similar clinical cases may have positive (but reduced) labelling of the dermo-epidermal junction using a different, purified, concentrated monoclonal antibody to the globular part of type VIIc,[13] suggesting that there may be some basement membrane zone expression of type VIIc, even in severe RDEB.

In vitro studies, primarily examining various epithelial cell lines, have suggested that type VIIc is mainly a product of epithelial cells, with a lesser additional contribution from fibroblasts.[4,8] There has been little *in vivo* evidence of type VIIc synthesis by keratinocytes apart from studies on grafted cultured epithelium.[14] The fine granular LH 7:2 fluorescence staining seen within the basal cells of normal human skin during wound healing in this study provides *in vivo* confirmation of keratinocyte type VIIc synthesis. In the severe RDEB skin samples, intraepidermal type VIIc fluorescence during wound healing is a new finding. This suggests that the keratinocytes in severe RDEB do have the capacity to synthesize or upregulate production of at least part of the type VIIc molecule, although exact quantification of synthesis is difficult to assess solely by immunofluorescence. In future, studies using antibodies to other parts of the collagen molecule will be necessary to assess further the completeness of type VIIc synthesis. Additionally, immunoblotting techniques could be used to assess the presence of type VIIc,[5] and the application of *in situ* hybridization using recently described c-DNA probes for type VIIc mRNA[15] would help to confirm or refute the immunofluorescence findings. Skin samples taken after the 2-week study period would also be necessary to comment more fully on the secretion of type VIIc.

The finding of intraepidermal type VIIc in severe RDEB during wound healing is somewhat different from observations noted in recent studies suggesting retention of type VIIc within the epidermis in a small number of dystrophic EB cases.[16–21] In those cases, the type VIIc abnormality was mostly seen as a transient phenomenon representing a temporary defect in intracellular processing, possibly calcium dependent,[22] or impaired secretion of the collagen molecule. In severe RDEB, a more permanent abnormality of type VIIc persists, which may be due to incorrect synthesis (molecular mutations of type VIIc), defective association with accessory macromolecules,[23] or excessive molecular breakdown (proteolytic enzyme activity)[24,25] or a combination of one or more of these potential defects. Intracellular type VIIc has also been observed in a proportion of well-differentiated squamous cell carcinomas arising in normal human skin,[26] suggesting that changes in keratinocyte differentiation (as occurs in a regulated fashion in wound healing) have a direct influence on the synthesis and expression of basement membrane components. Further work to determine the cellular growth factors and cytokines involved in promoting type VIIc synthesis (e.g. transforming growth factor-β)[9,27,28] under such circumstances may have some potential therapeutic relevance to dystrophic EB, particularly if a relative deficiency of type VIIc is the essential underlying abnormality. However, if a primary molecular type VIIc defect or abnormalities in secretion or polymerization of type VIIc underlie severe RDEB, sustaining growth factor expression may be inappropriate in attempting to modify this genodermatosis.

Acknowledgments

The assistance of Mr Mayou (Consultant Plastic Surgeon, St Thomas's Hospital) in providing skin samples is gratefully acknowledged. Bullous pemphigoid antiserum was kindly provided by Mr Bhogal (St John's Institute of Dermatology). This work was supported by grants from the Dunhill Trust and from the Dystrophic Epidermolysis Bullosa Research Association (DEBRA, U.K.). Dr McGrath is in receipt of a DEBRA research fellowship.

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British Journal of Dermatology (1992) 127, 318–321.

Epidermolysis bullosa in Northern Ireland

K.E.McKENNA, M.Y.WALSH* AND E.A.BINGHAM

*Departments of Dermatology and *Pathology, Royal Victoria Hospital, Belfast, Northern Ireland*

Accepted for publication 1 June 1992

Summary

Cases of epidermolysis bullosa (EB) diagnosed in Northern Ireland during a 23-year period (1962–84) were identified from dermatology clinic files, paediatric hospital notes and cases known by general practitioners. A total of 48 confirmed new cases of EB were diagnosed during the screening period. This involved 31 families, with identification of 36 further cases. The distribution of incident EB subtypes was: simplex 31 (65%), junctional 1 (2%), dystrophic 12 (25%) and acquisita 4 (8%). The incidence rate of new cases of EB diagnosed per year is 1·4/million and prevalence of all forms estimated at 32/million. The prevalence of simplex, junctional and dystrophic forms is 28, 0·7 and 3/million, respectively.

Epidermolysis bullosa (EB) is a heterogeneous group of genetic disorders characterized by marked skin fragility, blister formation, and in some cases by significant scarring. There are three major types of hereditary EB—simplex, junctional and dystrophic. Each type has certain characteristic clinical features and a specific site of cleavage within or adjacent to the epidermal basement membrane.[1–4] In addition, there is an acquired type of EB with characteristic immune deposition in the sublamina densa zone.[5]

Few studies have been performed regarding the epidemiology of EB.[6–11] We have performed an epidemiological study of the incidence and distribution of types and subtypes of EB occurring in Northern Ireland during a 23-year period.

Methods

Patients

Cases of EB diagnosed in Northern Ireland (NI) during the period 1962–84 inclusive were identified from dermatology clinic notes. This time interval was selected because records were complete for all clinics during this period. This included four Belfast hospital-based clinics, including the Royal Belfast Hospital for Sick Children, and 18 other clinics distributed throughout NI. Cases recorded in paediatric hospital notes were also noted. Patients were contacted to attend for review. In addition, general practitioners were contacted to assist in tracking cases.

Classification of EB cases

Diagnostic criteria for EB were similar to those used by Gedde-Dahl,[6] supplemented by electron microscopy (EM)[12] and immunofluorescence antigen mapping (IAM)[13,14] to determine the level of blister formation. The skin was examined for blister distribution, scarring, milia, pigmentary abnormalities, acral hyperkeratosis and cutaneous neoplasia. Involvement of mucous membranes, nails, teeth, eyes and hair was recorded. A family pedigree of each affected family was constructed. Further affected members or secondary cases were identified and interviewed if possible.

Skin biopsy

The clinical diagnosis was confirmed when possible by skin biopsy. Two 4-mm punch biopsies were taken from recent blisters, or rubbed skin, using local anaesthesia with 1% lignocaine. The samples were examined by EM and IAM. Antibodies used included those against bullous pemphigoid antigen, laminin, type IV and type VII collagen.

Results

A total of 72 new cases of EB were recorded during the screening period. Of these, 10 were not EB, and in 14 the notes had either been destroyed or lost. The remaining 48 cases had EB, and from these index cases or probands a further 36 secondary cases were identified from pedigree studies. This involved 32 families with one or more affected members.

Correspondence: Dr K E.McKenna, Department of Dermatology, Royal Victoria Hospital, Belfast BT12 6BA, Northern Ireland.

Table 1. Distribution of EB simplex subtypes. Skin biopsies examined by electron microscopy (EM) and immunofluorescence antigen mapping (IAM)

	Probands	Secondary cases	Families	EM/IAM
Koebner	3	0	3	—
Weber–Cockayne	26	34	17	4
Dowling–Meara	2	1	2	2
Mottled pigmentation	—	—	—	
Ogna	—	—	—	
Bart	—	—	—	

Simplex types

EB simplex (EBS) was diagnosed in 31 probands (Table 1). The most common variety was Weber–Cockayne (EBS–WC), with blistering affecting the palms and soles. This occurred in 26 patients, and a further 35 secondary cases were identified. The majority of EBS–WC cases occurred in two large families with 14 and 11 living affected members, respectively. Three patients had the Koebner form (EBS–K) in which disseminated blistering occurs. One also had dystrophic nails. Two cases of the Dowling–Meara form (EBS–DM) were recorded. Clinical features of EBS–DM included generalized blistering at birth with erosions and crusting. Blistering showed grouping and was non-scarring. Palmoplantar keratoderma developed in later life. Electron microscopic findings included basal cell cytolysis and clumping of tonofilaments. One patient is still resident in NI, the other lives abroad, and his son is affected. No cases with mottled pigmentation, and no Bart or Ogna forms of EBS were discovered.[15,16] A total of 30 affected individuals were examined and skin biopsy was performed in six patients.

Junctional types

Six main types of junctional EB (JEB) are recognized, and include the gravis, mitis, cicatricial, inversa, minimus and progressiva variants.[17] During our screening period one case of JEB of the inversa type was diagnosed in a male patient, who is still alive. The patient had a history of axillary and groin blistering since birth. Corneal involvement and nail dystrophy were also present.

Dystrophic types

A total of 12 dystrophic EB (DEB) index cases were identified (Table 2). These were represented by six dominant and six recessive cases. The four cases of Hallopeau–Siemens recessive DEB were related, and had died following the development of squamous carcinoma of the skin. Two other unaffected sibs had ichthyosis vulgaris. Interestingly, Gedde Dahl[6] reported three unaffected members in the family of a case of lethal DEB who had ichthyosis vulgaris. The two cases with localized recessive disease were brother and sister. Blistering mainly affected the limbs, and nails were dystrophic. Both patients complained of dysphagia, and upper oesophageal webs were demonstrated. Patients with autosomal dominant Cockayne–Touraine DEB[4] had blistering, and atrophic scarring with milia, occurring mainly over knees, elbows, fingers and toes. Nail dystrophy was present in three patients. Three of these cases were not traced and a secondary case had died of an unrelated illness. There was one case of Pasini DEB, with atrophy and scarring affecting mainly the limbs, with occasional truncal lesions. In addition, lumbar

Table 2. Distribution of dystrophic EB cases. Skin biopsies examined by electron microscopy (EM) and immunofluorescence antigen mapping (IAM)

	Probands	Secondary cases	Families	EM/IAM
Recessive				
Hallopeau–Siemens	4	0	1	—
Inversa	—	—	—	
Localized	2	0	1	1
Dominant				
Cockayne–Touraine	5	1	4	2
Pasini	1	0	1	1
Pretibial	—	—	—	

albopapuloid lesions were present. No cases of inversa or pretibial DEB were recorded.

A total of five cases of DEB were traced, and are resident in Northern Ireland. Four of these patients had skin biopsies performed.

Acquisita

Four patients with EB acquisita were diagnosed during the screening period. Unfortunately three of these patients are now dead, and the fourth cannot be traced. These patients were characterized by late onset of mainly acral blistering with milia formation. There was a negative family history of EB. The patients were not taking any systemic medications. Porphyria had been excluded by appropriate biochemical tests. Unfortunately these patients were diagnosed at a time when electron microscopy was not available in the province.

Incidence and prevalence

The incidence of newly diagnosed cases of EB occurring in Northern Ireland was calculated for the 23-year screening period. The population of Northern Ireland is approximately 1·5 million. The incidence rate of new cases of all types of EB is 1·4/million/annum. The incidence rate for EBS is 0·9, JEB 0·03 and DEB 0·3/million/annum. Of the 50 patients whose vital status could be ascertained on the 31 January 1991, 41 (82%) were alive and resident in Northern Ireland. In addition, a further seven cases (all simplex) were discovered after the screening period for which records are complete. A minimum estimate of the prevalence of all forms of EB in Northern Ireland is 32/million. The approximate prevalence of hereditary EB subtypes is EBS 28, JEB 0·7 and DEB 3/million. These figures are undoubtedly an underestimate as not all recorded cases were identified, and many cases with mild involvement go unreported.

Discussion

Several studies of the epidemiology of EB have been carried out. In Norway, Gedde-Dahl[6] estimated the minimum prevalence of all congenital types of EBS to be 24/million of population—EBS-K 1·1, EBS-WC 9 and EBS-Ogna 14·2/million of population. The prevalence of dominant DEB was 1·4 and recessive DEB 6·6/million. In Finland, Kero[7] identified cases of EB diagnosed over a period of 10 years. At the time of his study patients still alive included a total of 74 with EBS, one junctional EB, 41 dominant DEB, two recessive DEB and one EB acquisita. Kero concluded that the most significant difference compared with the Norwegian data was the much lower frequency of recessive dystrophic EB. Gedde-Dahl did not use ultrastructural criteria, hence junctional and dystrophic forms were classified in the same group. In the Oxford area of England, Davison[8] estimated the minimal frequency of all types of dominant EB together as 1/50,000 and for the recessive forms as 1/300,000. Winship[9] estimated the prevalence of EB in South Africa to be 1 in 354,195. Inaba *et al.*[10] estimated the prevalence of EB subtypes in Japan to be EBS 2·9–4, JEB 0·15–0·2, dominant DEB 1·1–1·5 and recessive DEB 1·5–2·1/million of population. Pavicic *et al.*[11] retrospectively analysed 27 years of records in Croatia. Fifty-eight patients with EB were discovered, 35 of whom were believed to have severe generalized DEB of the Hallopeau–Siemens variety. Seven patients were defined as having JEB, two dominant DEB, and only two EBS. The prevalence of EB in Croatia is estimated to be 0·956/100,000.

The prevalence of all types of EB in Northern Ireland is comparable with the disease prevalence in Norway. EBS and dominant DEB are more prevalent in Northern Ireland, but recessive DEB is less common. It is interesting to note the low prevalence of EB in Japan and Croatia, particularly the simplex form. Recessive DEB in Croatia appears to have a high incidence, and EBS to occur only rarely.

Epidemiological studies of EB are important, as patients can be located and their disease type classified accurately. This not only yields important statistical information, but can also serve as a data bank for continuing research into the pathogenesis of this heterogeneous group of disorders.

Acknowledgments

We thank Dr C.Patterson for statistical advice, Mrs J.Smith-Davidson and Miss E.King for assistance with tracing patients.

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British Journal of Dermatology (1992) 127, 322–327.

Exposure to house dust mites in homes of people with atopic dermatitis

M.J.COLLOFF
Department of Zoology, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Accepted for publication 4 June 1992

Summary

The median relative density of house dust mite populations was determined in mattresses in the homes of 23 people with atopic dermatitis (AD) who were mite-sensitive. It was significantly higher (total mites [dead and live] 174/0·1 g of dust; live mites 45·5/0·1 g) than in those of 23 healthy non-atopics (total mites 52/0·1 g; live mites 7·5/0·1 g; $P<0·05$, $<0·01$, respectively; Mann–Whitney *U*-test). There was no significant difference between mite population densities in mattresses in damp homes ($n=27$; total mites 188/0·1 g; live mites 36/0·1 g) and those in homes with no damp ($n=19$; total mites 116/0·1 g; live mites 26/0·1 g). These data demonstrate a clear association between AD and exposure to large numbers of mites, independent of the presence of domestic damp. People with AD were exposed to densities of >500 mites/0·1 g of mattress dust five times more frequently than non-atopics, corresponding to a relative risk of 25·0, but were exposed to densities of $\leqslant 50$ mites/0·1 g five times less frequently. The overall relative risk for AD, with a cutoff of 50 mites (total)/0·1 g, was 8·1. There was an obvious positive correlation between magnitude of exposure and frequency of disease. It is hypothesized that the association between high densities of mites and AD is related to differences in lipid composition of the shed squames on which the mites feed: those of people with AD may be of greater nutritional value than those of non-atopics, thus enhancing mite population growth.

House dust mites are the major source of airborne allergens in the home to which people with atopy are exposed.[1] There is compelling indirect evidence that house dust mites are important in the pathogenesis of atopic dermatitis (AD), including: high titres of allergen *Der*p_1-specific IgE antibody in most patients;[2] IgE antibody responses against a diversity of mite allergens amongst patients with AD;[3] marked clinical improvement following intensive eradication of mite allergens in the home[4] or removal of patients to allergen-free environments;[5] development of cutaneous basophil hypersensitivity and eczematous-type lesions following allergen patch-testing;[6,7] proliferation of T-cells from patients with AD *in vitro* in the presence of mite allergens;[8] the presence of mite allergens bound to Langerhans cells and Fc_ε-specific T cells after allergen patch-testing AD skin.[9]

These findings suggest that mite-sensitive people with AD receive a high degree of exposure to house dust mite allergens. Recently, Beck and Korsgaard[10] found that people with moderate/severe AD had significantly higher densities of mites in their homes than non-atopics or people with psoriasis.

The purpose of the present study was to investigate whether the homes of people with AD had significantly higher mite population densities than those of non-atopics, and whether there was any difference related to the presence of damp in the homes: humidity is a major limiting factor for the growth of house dust mite populations.

Methods

Patients and controls

Samples of dust from homes were taken during the run-in period before patients with AD embarked on a controlled clinical trial of house dust mite eradication.[11] Twenty-three adult patients (11 males, 12 females; age range 16–56 years) with symptomatic AD were recruited at the out-patient clinic of the Department of Dermatology, Western Infirmary, Glasgow. They all showed positive immediate skin prick-tests to *Dermatophagoides pteronyssinus* extract (Bencard Ltd.), and positive anti-*D. pteronyssinus* RAST scores, as well as elevated total serum IgE.[11] Twenty-three healthy non-

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This paper was presented, in part, at the Eighth International Congress of Acarology, České Budějovice, Czechoslovakia, 6–11 August, 1990.

atopics were selected from nominees of the author's acquaintances. They had no personal or family history of atopy or exfoliative skin disease. The sex ratio and age range of the group were similar to the group with atopic dermatitis (10 males, 13 females; age range 19–51 years). All members of both groups dwelt within Greater Glasgow, U.K., and none had previously conducted mite eradication procedures in their homes.

Dust sampling and mite extraction techniques

Single dust samples were taken from the mattress of each person between January and March, 1985, as follows: dust was collected from the surface of each mattress into a plastic sampling tube with a 10-μm mesh dust-trap, attached to a portable vacuum pump. The area from which dust was collected was standardized to 0·25 m^2 by using a zig-zag sampling pattern of 20 × 1 m long sweeps, taken in 1 min, with a sampling nozzle with an internal diameter of 12·5 mm.

Dust samples were weighed and placed, unsieved, into glass Petri dishes and macerated in 70% lactic acid. A few drops of 1% (w/v) aqueous lignin pink (BDH Ltd.) were added to stain the mites, and the dishes placed on a hotplate at 45°C for up to 3 h. The fluid was then poured off into a fresh dish. The first dish was scanned twice under a stereobinocular microscope and the mites removed from the remaining fluid with a finely drawn Pasteur pipette. The pouring process was repeated with the second dish, and so on, until all the fluid had been examined. Mites were washed in distilled water and mounted in Fauré's medium[12] using the frozen block technique.[13] Mean extraction efficiency was 81%. Mites were identified and counted under a compound microscope and were recorded as 'damaged' (i.e. dead) or 'intact' (i.e. live) using morphological criteria.[14]

Assessment of damp in homes

Homes were assessed visually and graded as 'damp' based on the presence of two or more of the following features: presence of mould growth on walls and ceilings, strong smell of mould, peeling and water-stained wallpaper and moist plasterwork, condensation on windows, walls and ceilings of rooms other than bathrooms or kitchens, and signs of direct water penetration. Homes graded as 'dry' had none of these features.

Table 1. The frequency of damp in homes of patients with atopic dermatitis and homes of healthy non-atopics

	Atopic dermatitis	Non-atopic	Total homes
Damp homes	13	14	27
Dry homes	10	9	19
Total homes	23	23	46

Statistical analyses

Mite population densities were expressed as numbers of mites per 0·1 g of dust. Medians were used as the measurement of central tendency, with interquartile ranges. Analysis of differences in mite population density was performed using the Mann–Whitney *U*-test. Analysis of differences in frequency of damp in homes, and numbers of people in each mite exposure class was performed using the χ^2 test with Yates' correction. Relative risk was calculated as described by Armitage.[15]

Results

Frequency of domestic damp

The frequency of damp in homes of patients with atopic dermatitis (57%) and homes of healthy non-atopics (61%) was not significantly different ($\chi^2 = 0{\cdot}18$; Table 1). In total, 59% of homes were graded as damp.

Mite population densities and atopic dermatitis

The median density of total mites (live and dead)/0·1 g dust from mattresses of people with AD was 174 (interquartile range [IQR] 86–451), 3·4 times higher

Table 2. Frequency of exposure to different population densities of house dust mites for people with atopic dermatitis and healthy non-atopics

Mite density	Atopic dermatitis	Non-atopic
Total mites/0·1 g of dust		
⩽50	2	10
51–500	16	12
>500	5	1
Live mites/0·1 g of dust		
⩽10	1	10
11–100	15	7
>100	4	1

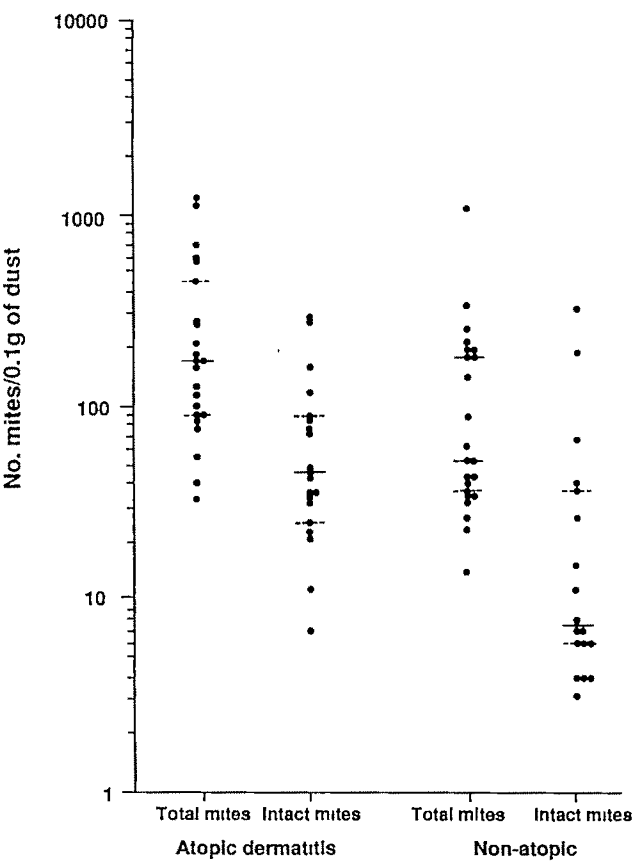

Figure 1. Scatterplot showing the densities of total mites (live and dead, all species) and intact mites (live) per 0·1 g of dust from mattresses of patients with atopic dermatitis and healthy non-atopics. Bars indicate 75th percentile, median, and 25th percentile. Live (intact) mites were detected in 20/23 homes of people with AD and 18/23 homes of non-atopics.

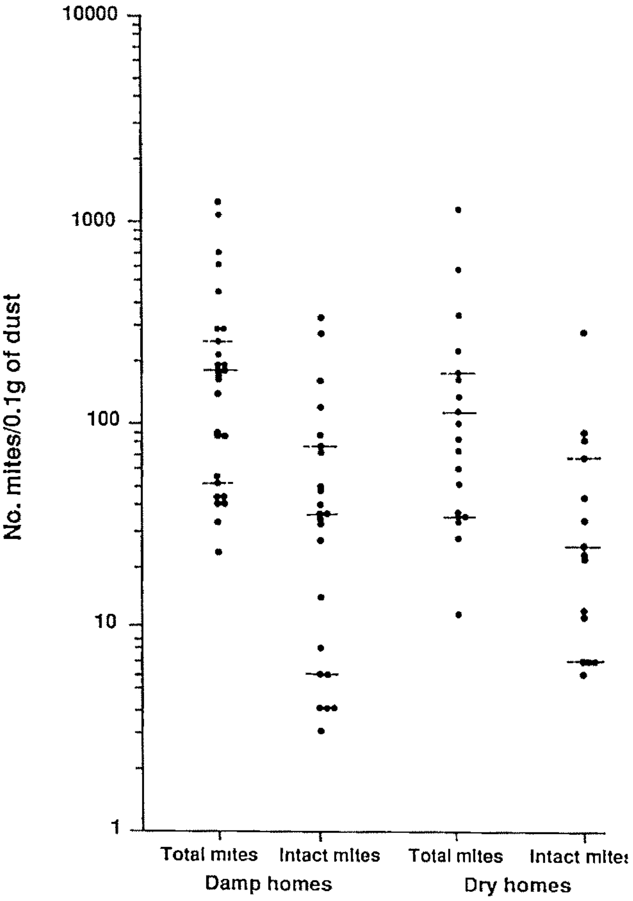

Figure 2. Scatterplot showing densities of total mites (live and dead, all species) and intact mites (live) per 0·1 g of dust from mattresses in damp homes and dry homes. Bars indicate 75th percentile, median and 25th percentile. Live (intact) mites were detected in 23/23 damp homes and 15/23 dry homes.

than that of non-atopics (52 mites/0·1 g; IQR 35–196; $P < 0{\cdot}05$ [Fig. 1]). The density of live mites was 6·1 times higher in mattresses of the AD group (46/0·1 g; IQR 26–88) than those of the non-atopic group (8/0·1 g; IQR 6–36; $P < 0{\cdot}01$ [Fig. 1]).

Total mite and live mite densities were each divided into three 'exposure classes' (Table 2). There were five times more non-atopics than people with AD exposed to densities of ≤50 total mites/0·1 g, 1·3 times more people with AD exposed to densities of 51–500/0·1 g and five times more exposed to densities of >500/0·1 g ($\chi^2 = 20{\cdot}8$; $P < 0{\cdot}001$). For live mites, people with AD were 10 times less likely than non-atopics to receive exposure to ≤10 mites/0·1 g, 2·1 times and four times more likely to be exposed to 11–100/0·1 g and >100/0·1 g, respectively ($\chi^2 = 16{\cdot}4$; $P < 0{\cdot}001$).

The overall relative risk for AD, with a cutoff of 50 (total) mites/0·1 g, was 8·1. Increased relative risk with increased exposure, i.e. a dose-response relationship, was demonstrated both for total and for live mites (Fig. 3).

Mite population densities and domestic damp

The median density of total mites and live mites in mattresses in homes with damp was 188/0·1 g (IQR 52–250) and 36/0·1 g (IQR 6–76), respectively, and 116 total mites/0·1 g (IQR 34–170) and 26 live mites /0·1 g (IQR 7–68) in mattresses in dry homes. Although damp homes had slightly higher median mite densities than dry ones, they were not statistically significantly different (Fig. 2).

Discussion

Mattresses of a group of mite-sensitive patients with atopic dermatitis had a significantly higher median mite

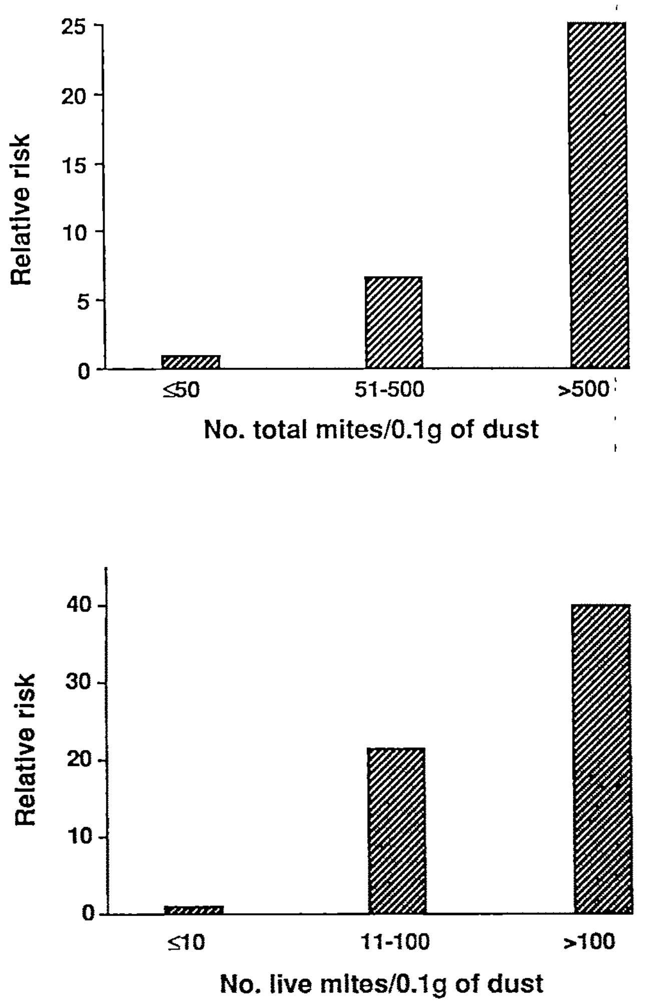

Figure 3. Histogram showing relative risks of patients having atopic dermatitis according to the densities of total and live house dust mites in their mattresses. For total mite relative risk, $n = 23$ people with AD, 23 non-atopics; for intact mite relative risk $n = 20$ people with AD, 18 non-atopics. Relative risk values are: total mites 51–500, 6·7; >500, 25·0; live mites 11–100, 21·4; >100, 40·0.

population density than those of a group of healthy non-atopics, independent of domestic damp. The association between atopic dermatitis, dense house dust mite populations, and heavy exposure to mite allergens, confirms the findings of Beck and Korsgaard[10] and Harving *et al.*,[16] and leads to the interpretation that increased exposure to mites could be the cause of AD in adult patients. But if people with atopy develop eczematous lesions because they live in homes where they are exposed to high mite densities, then why do these homes have such high densities in the first place?

It has been shown herein that domestic damp is not a factor associated with high mite population densities in mattresses. Using visual assessment as a means of assessing domestic damp is open to criticism because it can be difficult to distinguish between damp caused by bad insulation of the roof and outer walls—which may not necessarily be accompanied by high indoor humidity or increased mite population growth—and damp associated with higher indoor humidity caused by water vapour-generating activities within poorly ventilated homes, which would encourage mites. However, in the present study, 85% of damp homes had mould growth, indicating high indoor humidity due to poor ventilation. In temperate latitudes, mites in mattresses receive massive diurnal fluctuations in microclimate. Depending on seasonal macroclimate, for about a third of the day they are exposed to conditions of ca. 30–35°C, 80–95% r.h. due to heat and moisture generated by the occupant(s) of the bed. When the bed is vacated, temperature and humidity gradually fall and reach equilibrium with the bedroom air, typically around 18°C, and below 55% r.h.[17] When humidity falls too low, mites dehydrate and eventually die. It would appear that in the present study the humidity during daylight hours in bedrooms of dry homes was high enough not to limit mite population growth.

If high densities of mites in homes of people with AD are not due to damp, is it possible that features of the disease itself can lead to high mite densities? My hypothesis is that certain characteristics of AD would be beneficial to house dust mites and could promote development of, or sustain, large mite populations. This would create a positive feedback mechanism: as larger populations develop, so exposure to allergens becomes more severe and skin lesions become progressively worse. In this scheme, mite population growth would be down-regulated by episodic low temperature and humidity, even in normally humid homes, caused by the cool dry winter climate that prevails in temperate northern latitudes. The hypothesis necessarily implies that transcutaneous sorbtion of mite allergens takes place in people who develop AD,[18] and that skin is sufficiently dry to allow allergen ingress before the development of eczematous lesions. Indeed, a generalized xerosis is characteristic of AD and can be present without gross lesions.[19]

There are two phenomena associated with AD that may promote high mite populations. First, there is increased transepidermal water loss[20,21] which may promote a more humid environment than in mattresses of non-atopics. Second, there is the lipid composition of the squames. Beck and Bjerring[22] suggested that the copious shedding of squames (on which the mites feed) by people with AD may enhance mite population growth, but Beck and Korsgaard[10] found there was no difference in mite population density between homes of people with psoriasis and healthy controls. However, it has been suggested[23] that the nutritional quality of squames is far from homogeneous. Lipid composition and concentration are important determinants of mite population growth.[24] Certain fatty acids impair mite development,[25] and fresh squames from healthy non-atopic skin, with a relatively high total lipid content, can actually inhibit mite population growth.[24,26] Squames from people with AD have lower total lipid concentrations than those of non-atopics and people with psoriasis, as well as markedly different concentrations of specific lipids.[21,27] These differences are linked with impaired epidermal barrier function due to disrupted biosynthesis of ceramides, so that lipid lamellae in the stratum corneum do not form properly.[27] Thus, squames from people with AD may be of greater nutritional value to house dust mites than those of healthy non-atopics because: (a) the stratum corneum lipids are only partially assembled and easier to digest than fully assembled lipid lamellae; (b) the total lipid content may match more closely the dietary optima for the mites; (c) the squames contain lower concentrations of those free fatty acids that inhibit mite population growth.

The high exposure to house dust mites in mattresses received by patients with AD in this study, together with an obvious dose-response relationship, reinforces the epidemiologcal evidence that mite allergens are a cause of AD. It follows that in mite-hypersensitive patients with AD, symptoms should improve following reduced exposure to mite allergens. Bedding covers are a most effective means of isolating people from the allergens in their mattresses, and may well be of clinical benefit to patients with AD.

Acknowledgments

This work was funded by grants from the Medical Research Council and the Wellcome Trust. I thank Dr Jens Korsgaard (Department of Respiratory Diseases, University Hospital of Aarhus) for advice on data analysis and for helpful criticisms of the manuscript, and Dr Rosemary Lever (Department of Dermatology, Western Infirmary, Glasgow) for help with clinical aspects of the study.

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Increased expression of tenascin in the dermis in scleroderma

J.P.LACOUR,* A.VITETTA,* R.CHIQUET-EHRISMANN,‡ A.PISANI† AND J.P.ORTONNE*†

**Department of Dermatology, and †Laboratory of Dermatologic Research, University of Nice, Nice, France*
‡Friedrich Miescher Institute, Basel, Switzerland

Accepted for publication 29 April 1992

Summary

The expression of tenascin, a recently discovered extracellular matrix protein, was studied by immunohistochemical techniques in scleroderma skin and compared with its distribution in normal skin. In progressive systemic sclerosis, a marked increase in tenascin content was observed in the superficial reticular dermis. In localized scleroderma, the deposition of tenascin was increased both in the superficial and deep dermis of involved skin, whereas in clinically uninvolved skin the distribution of tenascin was the same as in normal control skin, i.e. the papillary dermis and periappendageal zone. The distribution of tenascin did not strictly parallel that of fibronectin. These findings and the current knowledge of tenascin biology suggest that the overproduction of tenascin in scleroderma dermis could be secondary to stimulation of fibroblasts by immune cell-derived cytokines, or could be due to abnormal fibroblasts, or a subpopulation of fibroblasts, producing high levels of this extracellular matrix protein.

Scleroderma is a connective tissue disease characterized by fibrosis of the skin and, in the progressive systemic form, internal organs.[1] The prominent cutaneous histological change is an increase in collagen fibres in the reticular dermis and in the subcutaneous tissue. *In situ* hybridization studies have shown an increased expression of collagen genes in affected tissues from patients with systemic sclerosis (SSc)[2] and *in vivo* studies of skin fibroblasts have shown increased collagen synthesis per cell.[3,4] Other modifications of dermal components accompany the change in collagen content. There are changes of the dermal matrix, with an increase in glycosaminoglycans.[5] There is also a marked increase of fibronectin in the deep dermis, whereas the papillary dermis fibronectin content is unchanged.[6,7] Thus, an as yet unknown primary event appears to lead to fibroblast dysfunction, resulting in increased extracellular matrix production.

Tenascin is a recently recognized high molecular weight glycoprotein produced by mesenchymal cells. It is present in several organs during the fetal period, being involved in epithelial–mesenchymal interactions during morphogenesis.[8] Although the content of tenascin decreases in several tissues during the post-natal period, it is still present later in some sites. In adult human skin, for example, tenascin is located in the papillary dermis and near the basement membrane of sweat glands.[9,10] Immunohistochemical study of the wound healing process, has shown particularly strong tenascin staining beneath the migrating and proliferating epidermal cells.[11] The exact function of this extracellular matrix is still not clear. Tenascin often co-localizes with fibronectin in tissues, and it has been shown that tenascin interferes with fibronectin action on cell attachment and spreading.[12–14]

In this study, we investigated the distribution of tenascin in localized and SSc skin, compared with normal skin samples. We have shown by immunofluorescence techniques that tenascin is markedly increased in the superficial reticular dermis of SSc and in the entire reticular dermis of localized scleroderma. The distribution of tenascin did not strictly parallel that of fibronectin.

Methods

Patients and biopsy specimens

Six-millimetre punch biopsy specimens were obtained from 14 patients with SSc, 12 patients with localized scleroderma, and six healthy adult volunteers. The

Correspondence: Dr J-P.Lacour, Department of Dermatology, Hôpital Pasteur, 30 Voie Romaine, BP 69, 06002 Nice Cedex, France.

Table 1. Clinical characteristics of the SSc patients studied

Case no.	Age/sex	Years of disease	Clinical picture	Site of biopsy
1	40/F	6	R, S, T, A	Forearm
2	60/F	13	C, R, O, S, T	Forearm
3	63/F	8	C, R, O, S, T, GS	Forearm
4	67/F	6	R, O, S, T	Forearm
5	70/F	15	R, S, T	Forearm
6	56/F	10	R, S, T, A	Forearm
7	22/M	12	C, R, S, T, P, GS	Forearm
8	56/F	15	C, R, O, S, T, P	Forearm
9	74/F	15	R, O, S, T, P	Forearm
10	63/F	4	R, S, M, A	Forearm
11	63/F	2	R, O, S, T	Neck
12	66/F	4	R, O, S, T, P	Forearm
13	79/F	7	R, O, S, T, GS	Forearm
14	61/F	10	R, S, T, A	Forearm

S, sclerodactyly; R, Raynaud's phenomenon; O, oesophageal involvement; C, calcinosis; T, telangiectasia; P, pulmonary involvement; M, myositis; A, arthritis; GS, Gougerot–Sjögren syndrome.

clinical data of the SSc patients are shown in Table 1. The biopsies were taken from clinically involved skin, usually the volar aspect of the forearm. In localized scleroderma patients, the biopsies were taken both from involved skin and adjacent uninvolved skin. The clinical characteristics of these patients are summarized in Table 2. Normal skin samples were obtained from the forearms of healthy adult volunteers. All biopsy specimens were immediately frozen in liquid nitrogen and stored at −80°C until use.

Antibodies

The anti-tenascin antiserum was provided by R.Chiquet-Ehrismann, Friedrich Miescher Institute, Basel, Switzerland. This antiserum, whose characteristics and specificity have been described elsewhere,[8] was prepared in rabbits against purified tenascin obtained from conditioned medium of chick embryo fibroblast culture.

Indirect immunofluorescence

Four-micrometre thick cryostat sections from the skin specimens were air-dried, and fixed in acetone at 4°C for 10 min. The slides were washed twice with phosphate-buffered saline (PBS) for 10 min, then incubated for 1 h at room temperature with the anti-tenascin antiserum diluted 1:100 in PBS. After two 10-min washes with

Table 2. Clinical data of the localized scleroderma patients studied

Case no.	Age/sex	Years of disease	Clinical picture	Biopsy site
1	61/F	20	GM	Thigh
2	54/M	2	MM	Abdomen
3	72/M	0·3	MM	Arm
4	40/M	10	PM	Back
5	42/F	0·5	MS	Arm
6	77/F	0·4	MM	Thigh
7	51/F	1	SM	Buttock
8	58/F	10	MM	Thigh
9	25/F	1	MM	Thigh
10	61/F	10	MM	Leg
11	45/M	1·5	PM	Forearm
12	63/F	6	MM	Arm

GM, generalized morphoea; MM, multiple plaques of morphoea; PM, pigmented morphoea; MS, monomelic scleroderma, SM, single plaque of morphoea.

PBS, the sections were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit gammaglobulin (Dakopatts, Denmark) diluted 1:30 in PBS. After two 10-min washes in PBS, the sections were counterstained with propidium-iodide, rinsed again with PBS, and mounted with glycerol-paraphenylenediamine. The slides were examined under a Zeiss fluorescence microscope.

Immunoperoxidase technique

Some of the skin samples were also processed by an immunoperoxidase technique. The first steps of the method were the same as for immunofluorescence. The second incubation utilized a swine anti-rabbit gammaglobulin antiserum conjugated with horseradish peroxidase (Dakopatts, Denmark). Diaminobenzidine was used as a chromogenic substrate.

Results

Normal skin

Immunohistochemical staining of the skin samples from control subjects showed that tenascin was mainly concentrated in the papillary dermis, close to the dermo-epidermal junction (Fig. 1). The staining appeared as a thin, homogenous linear band, but was thicker in the dermal papillae. This pattern was identical in all six

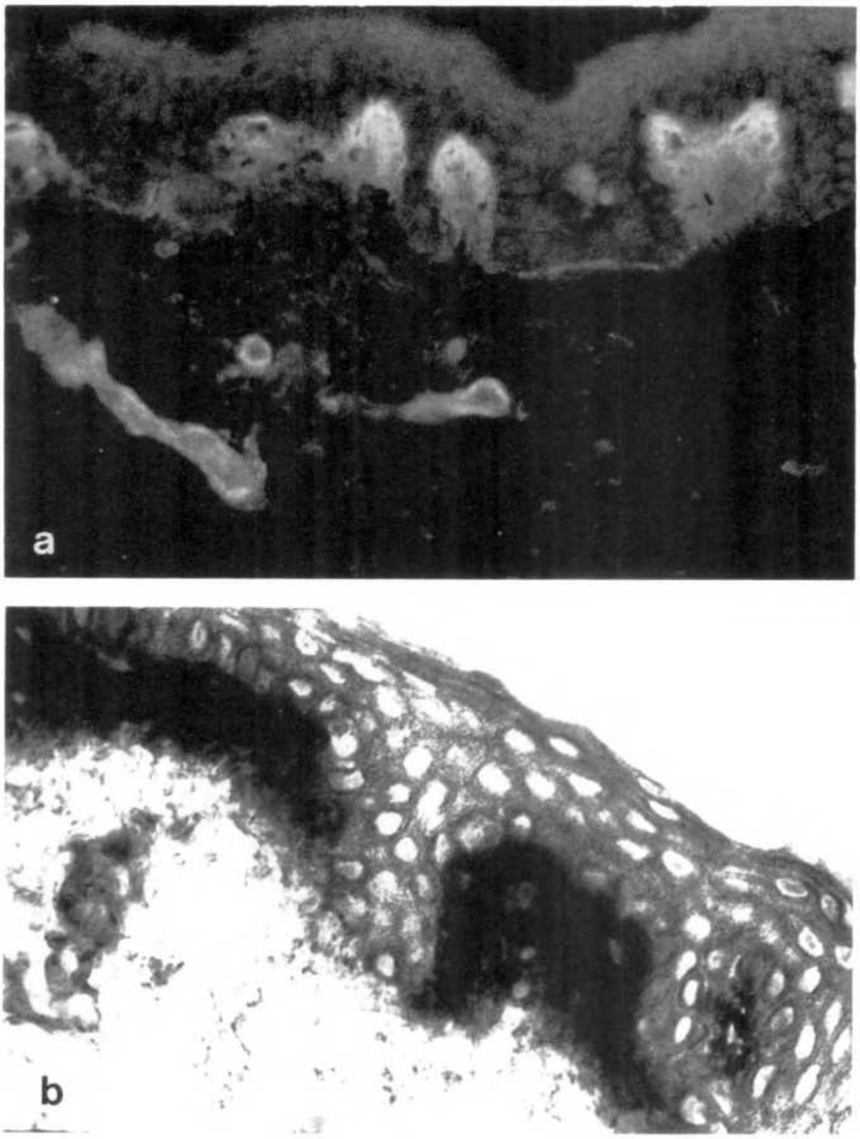

Figure 1. Photomicrographs of biopsy specimen of normal control skin immunohistochemically stained with anti-tenascin antibody. Tenascin is concentrated beneath the dermal–epidermal junction. The staining appears thicker in the dermal papillae. (a) Immunofluorescence technique (×250). (b) Immunoperoxidase technique (×400).

control skin specimens studied. No staining was observed in the reticular dermis. Similar staining was also observed around the hair follicles, the sweat glands and the dermal vessels.

SSc skin

Staining was observed in the papillary dermis and in the superficial part of the reticular dermis. It was more intense and thicker than in control skin (Fig. 2). The staining appeared stronger at the top of dermal papillae, as in normal skin specimens. However, dermal papillae were less visible than in normal skin because of the epidermal atrophy and flattening of rete ridges commonly present in scleroderma skin. The staining of the hair follicles, sweat glands and vessels was not modified. This pattern was very similar in all 14 SSc skin specimens studied.

Localized scleroderma—clinically involved skin

A marked increase in tenascin staining was observed in the upper dermis of involved skin, as in SSc skin. Moreover, in most of the morphoea skin sections examined, a diffuse and strong staining was observed throughout the reticular dermis, including deep dermis (Fig.3a). The staining of the hair follicles, sweat glands and vessels was identical with control normal skin. This pattern was similar in all 12 localized scleroderma skin specimens studied.

Clinically normal skin from localized scleroderma patients

The results were similar to those obtained with normal human skin (Fig. 3b).

Discussion

Our results show a marked increase in tenascin content in the reticular dermis of involved scleroderma skin. In normal skin, the tenascin distribution is known to be restricted to the superficial papillary dermis.[9] Our study confirmed these previous findings. The immunohistological analysis of tenascin distribution is not precise enough to establish whether or not it is a component of the dermal–epidermal junction. By immunoelectron microscopy we have observed that tenascin is exclusively present in the sub-basement membrane region of the dermis, sparing the dermal–epidermal junction itself (data not shown).

In SSc the increase in tenascin expression appeared to be confined to the superficial part of the reticular dermis, whereas in localized scleroderma skin the increase extended throughout the entire dermis. We cannot establish from this study whether such a difference reflects a dissimilar pathogenesis of these forms of scleroderma or is simply secondary to variations in intensity of dermal sclerosis. The absence of objective quantitative methods for clinical evaluation of cutaneous sclerosis and immunohistological dermal tenascin content does not allow a precise correlation between clinical and morphological data. However, the pattern of

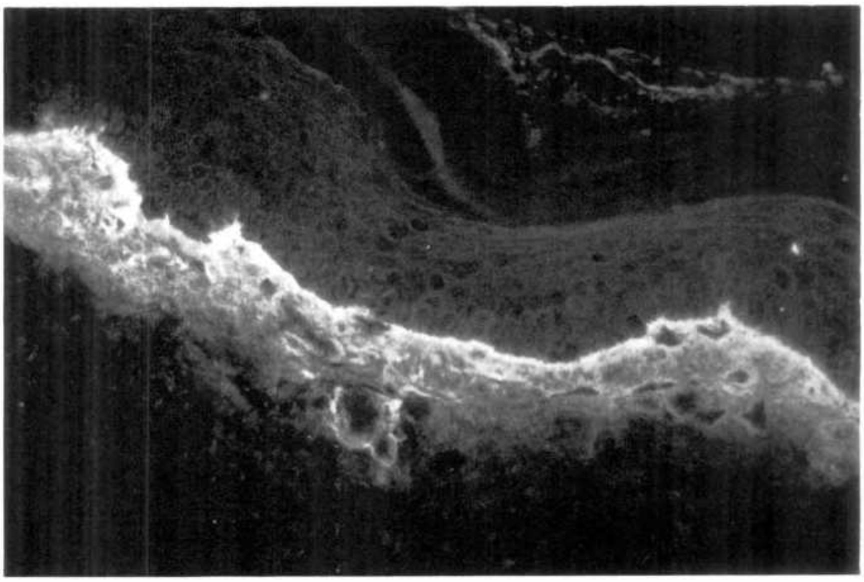

Figure 2. Indirect immunofluorescence staining of SSc skin with antibody to tenascin. The staining is thicker than in normal skin. (× 250).

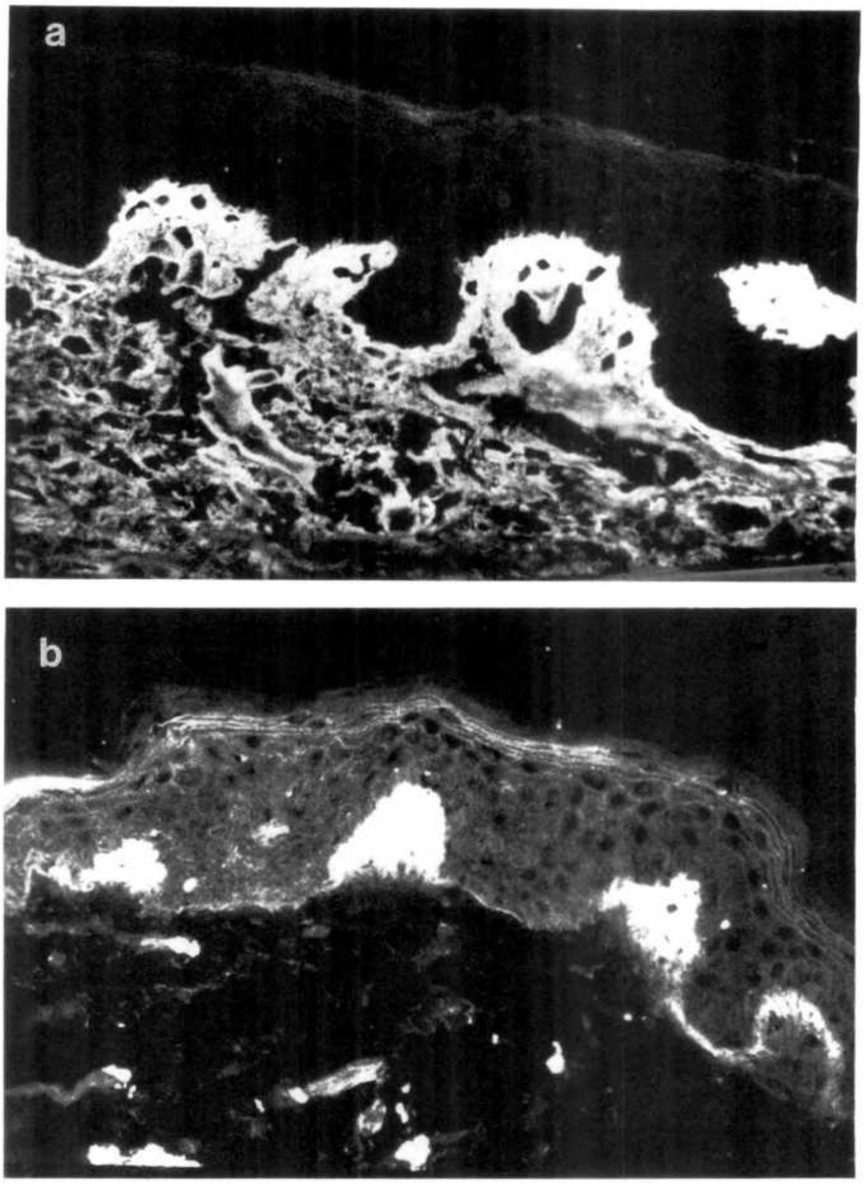

Figure 3. Indirect immunofluorescence staining with antibody to tenascin: (a) localized scleroderma—clinically involved skin: intense staining of the entire dermis. (b) clinically normal skin from localized scleroderma patient: the staining pattern is similar to normal human skin. (× 250).

tenascin distribution was uniform within each of the three groups.

Tenascin is a high molecular weight glycoprotein, consisting of six disulphide-bound subunits, synthesized by fibroblasts and muscle cells.[8] Tenascin can bind to proteoglycans and fibronectin, and inhibits *in vitro* both attachment and spreading of fibroblasts, interfering with fibronectin action.[12–14] Although its expression and role seem to be predominant during fetal development,[8] wound healing[11] or transformation,[8,15] tenascin appears to be a consistent, although variably distributed, component of the normal and pathological periepithelial stromal regions.[16] In the skin and in the breast, tenascin is present under normal circumstances, but is increased in pathological (benign or malignant) conditions characterized by epithelial hyperplasia.[10,16] In the skin, for example, an increase in tenascin is observed in the dermis of psoriasis lesions, in the stroma surrounding basal cell carcinomas,[10,17] and under the migrating and proliferating epidermal cells during wound healing.[11] Thus, the expression of tenascin appears to be in some circumstances the result of epithelial–mesenchymal interactions, and it is possible to hypothesize that there is an epithelial modulation of tenascin synthesis by fibroblasts. Cytokines derived from keratinocytes could be responsible for this fibroblast regulation. This does not exclude the possibility that tenascin synthesis might be regulated by other cytokines, derived from dermal inflammatory cells. In heavily infiltrated follicles in alopecia areata, for example, an increased perifollicular expression of tenascin has been observed, whereas this was not seen in areas in which a substantial infiltrate was not present.[18] In the same way, the inflammatory dermal infiltrate of psoriasis, and the inflammatory cells of cancer stromal reaction could be responsible for the modulation of tenascin expression by mesenchymal cells. In scleroderma, some of the change in the metabolism of fibroblasts, such as an increase in tenascin synthesis, could be the result of stimulation by cytokines derived from lymphocytes and macrophages observed in the skin of patients with recent-onset scleroderma. It has been suggested that mononuclear cells infiltrating the dermis play a role in stimulating the fibrotic process by influencing fibroblast metabolism.[19] Recently, an increase in intercellular adhesion molecule-1 expression (ICAM-1) in SSc has been demonstrated, and it has been suggested that this phenomenon is due to a stimulatory effect of cytokines generated by mononuclear cells.[20] Among these cytokines, transforming growth factor-β (TGF-β1) is known to induce the expression of tenascin.[21] However, although TGF-β1 expression has been demonstrated in dermal infiltrating cells and fibroblasts in generalized morphoea, it has not been detected in the dermis in SSc.[2] Finally, tenascin could be synthesized in response to immune dysfunction because it has been shown that it exerts *in vitro* immunosuppressive activity.[22]

In addition to this immune theory for scleroderma, there is the possibility that fibroblasts, or a subpopulation of abnormal fibroblasts, might be able spontaneously to produce excessive amounts of type I and III procollagen, fibronectin, and glycosaminoglycans, leading to fibrosis. Thus, tenascin could be over-synthesized like other extracellular matrix components.

The reason why fibronectin and tenascin distribution in scleroderma dermis is not strictly parallel is not known. These two different extracellular matrix components are often co-localized in tissues.[13] We have shown in this study that in SSc the increase in tenascin was limited to the superficial part of the reticular dermis, whereas fibronectin has been shown to be increased in the deep dermis of involved scleroderma skin.[6,7] However, these studies have been conducted using antibodies that could not distinguish between cellular and plasma fibronectin. Thus it would be important to re-evaluate the distribution of fibronectin in scleroderma dermis using antibodies to cellular and plasma fibronectin,[16] and to distinguish between *in situ* dermal production of fibronectin and dermal accumulation secondary to the vascular abnormalities of scleroderma.

In conclusion, our study shows that there is an increased expression of tenascin in scleroderma skin. The synthesis of tenascin by fibroblasts might be stimulated by immune cell-derived cytokines. However, we cannot establish whether the increase in tenascin is a primary event in scleroderma pathogenesis or simply reflects the exuberant connective tissue biosynthesis observed in this disease.

Acknowledgments

We thank Dr K.Werninghaus for checking the English version of the manuscript. Special thanks to L.Giordano for preparing the manuscript.

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British Journal of Dermatology (1992) 127, 335–343.

Adhesion molecules and related proteins in Darier's disease and Hailey–Hailey disease

S.M.BURGE AND K.H.SCHOMBERG
Department of Dermatology, Slade Hospital, Oxford

Accepted for publication 1 June 1992

Summary

We have used antibodies to plakoglobin and E-cadherin: the lectins, peanut agglutinin (PNA) and soybean agglutinin (SBA); and sera from patients with the autoimmune diseases pemphigus vulgaris (PV) or pemphigus foliaceus (PF), in an immunohistological study of Darier's disease and Hailey–Hailey disease. There was normal expression of plakoglobin, E-cadherin, lectins and pemphigus antigens at the periphery of keratinocytes in uninvolved skin. Clumps of plakoglobin were detected within acantholytic cells in Hailey–Hailey disease, whereas expression was diffuse in acantholytic cells in Darier's disease. This difference may reflect differences in the pathogenesis of acantholysis. E-cadherin expression was weak or absent at the periphery of some acantholytic cells; lectin binding was sometimes reduced around acantholytic cells, and pemphigus antibodies did not bind to the acantholytic cells in involved skin in either disease. Internalization, conformational changes or proteolysis may alter the expression of extracellular epitopes by acantholytic cells.

Darier's disease and Hailey–Hailey disease are dominantly inherited dermatoses in which there is abnormal adhesion between keratinocytes.[1] Interactions between the extracellular domains of cell adhesion molecules mediate adhesion and there may be abnormalities in the production, structure or function of an adhesion molecule in these diseases.

Two types of specialized intercellular adhesion junctions have been described in the epidermis, desmosomes (*macula adherens*) and intermediate or adherens junctions (*zonula adherens*).[2,3] Adhesion between keratinocytes is mediated by transmembrane glycoproteins which are present in both types of junction. E-cadherin, identical to uvomorulin or L-CAM, is probably the major adhesion molecule found in adherens junctions.[4] Adhesion molecules in the desmosomes include desmosomal glycoprotein I (desmoglein, dg1) of which there may be several subtypes,[5] and a pair of related glycoproteins, desmosomal glycoprotein 2 (dg2) and desmosomal glycoprotein 3 (dg3), also known as the desmocollins.[2] Like cadherins these desmosomal glycoproteins (dgs) are calcium-binding, transmembrane proteins. Cloning and sequence analysis has revealed that the extracellular domains of dgs and cadherins show extensive homology, supporting the view that desmosomal glycoproteins play a role in cell-to-cell adhesion in the desmosomal junctions.[6–8]

The submembranous junctional plaques in adherens junctions and desmosomes contain a number of non-glycosylated proteins, which may regulate adhesion by interacting with cytoplasmic portions of the transmembrane adhesion molecules. Desmosomal protein 3 (dp 3, plakoglobin, M_r 83 kDa) is the only plaque protein known to be present in both adherens junctions and desmosomes.[9] Interactions between dp3 and adhesion molecules may regulate adhesion in both types of junction.[10–12]

In a previous study of Darier's disease and Hailey–Hailey disease, we examined the expression of the major components of desmosomes. Dg1 and dg2/3 were internalized in acantholytic cells but there were some differences in the distribution of internalized components in the two diseases.[13] In the present study we have determined the immunolocalization of components of adherens junctions (dp3 and E-cadherin) and some non-junctional adhesion molecules. Sera from patients with the autoimmune blistering diseases pemphigus vulgaris (PV) and pemphigus foliaceus (PF) were used to examine the distribution of the extracellular portions of some adhesion molecules: PV antibodies bind to the extracellular portion of a 130–140-kDa transmembrane glycoprotein which shows homology to dg1 but is expressed uniformly on the cell surface, and is not restricted to

Correspondence: Dr S.M.Burge, Department of Dermatology, Stoke Mandeville Hospital, Aylesbury, U.K.

desmosomes,[14,15] while PF antibodies bind to an extracellular domain on dg1.[16]

Extracellular carbohydrate residues were also studied using the plant lectins, peanut agglutinin (PNA) and soybean agglutinin (SBA). Immunogold studies have demonstrated that SBA, which interacts with members of both the 150-kDa (dg1) and the 97–118-kDa (dg2/3) families, binds to desmosomes, while PNA, which binds preferentially although not exclusively to the 150-kDa (dg1) family, is localized predominantly to the plasma membrane between desmosomes.[17,18]

Methods

Patients and tissue

The protocol was approved by the Central Oxford Research Ethics Committee, and informed consent was obtained from the patients. Six unrelated adults with histologically confirmed Darier's disease (five males and one female); and nine adults with histologically confirmed Hailey–Hailey disease (six males and three females from five families) were included in the study. Punch biopsies 4 mm in diameter were obtained from typical lesions. The biopsies included perilesional uninvolved skin. Four specimens of normal skin collected during routine surgical procedures were used as controls. The biopsies were snap-frozen in liquid nitrogen. Frozen sections (8 μm) were cut and stored at −70°C.

Antibodies

The details of the antibodies used are shown in Table 1. A polyclonal antibody to E-cadherin was provided by Professor S. Tsukita, National Institute for Physiological Sciences, Moyodaiji, Japan. A monoclonal antibody to dp3, clone PG 5.1, was purchased from Cymbus Bioscience Ltd, 2, Venture Rd., Chilworth Centre, Southampton, Hampshire. Monoclonal antibodies to cytoplasmic domains of dp 1/2 (11-5F), dg1 (33-3D) and dg2/3 (52-3D) were provided by Professor D.R.Garrod, Cancer Research Campaign Medical Oncology Unit, University of Southampton, Southampton General Hospital, Southampton, U.K. The characteristics of these antibodies have been described in a previous study.[13]

Lectins

FITC-conjugated PNA and SBA were obtained from Vector Laboratories Ltd, 16 Wulfric Square, Peterborough, Cambridgeshire.

Human sera

Pemphigus sera were obtained from patients with the typical clinical, histological and immunological features of either PV (2) or PF (2). The pemphigus sera contained IgG antibodies which bound to the cell surface of keratinocytes in normal human epidermis, but did not bind to the basement membrane zone (BMZ).

Bullous pemphigoid (BP) serum containing IgG antibodies which bound in a linear band to the BMZ was used as a control. Bullous pemphigoid is an autoimmune disease in which the antigens are intracellular hemidesmosome-associated proteins which are expressed in the BMZ and are structurally similar to dp1 and dp2.[19]

Normal human serum was obtained from the Oxford Blood Transfusion Service.

Immunostaining

Monoclonal and polyclonal antibodies

Anti-E-cadherin was diluted 1:500 in phosphate-buffered saline (0·05 mol/l phosphate, NaCl 0·15 mol/l buffer, pH 7·4; PBS) with 1% BSA. Before staining with anti-E-cadherin, sections were fixed in 95% ethanol for 30 min at 4°C, followed by acetone for 1 min at room temperature, and washed in PBS with 1% BSA. Non-specific binding was blocked by pre-incubation in PBS

Table 1. Details of antibodies

Catalogue number or designation	Ig subclass	Specificity	Source or reference	Dilution
11-5F	IgG1	dp1 + 2 (desmoplakins)	Burge, Garrod, 1991[13]	1:20
33-3D	IgM	dg1 (desmoglein)	Burge, Garrod, 1991[13]	1:2
52-3D	IgG1	dg2 + 3 (desmocollins)	Burge, Garrod, 1991[13]	1:10
CBL175	IgG2b	dp3 (plakoglobin)	Cymbus Bioscience Ltd.	1:50
Anti-E-cadherin	Polyclonal	E-cadherin	S Tsukita (personal communication, 1991)	1:500

with 1% BSA for 10 min. PBS with 1% BSA was used for all washes.

Monoclonal antibodies to the desmosomal components dp1/2 (11-5F), dp3, dg1 (33-3D) and dg2/3 (52-3D) were used on unfixed material. Optimum working dilutions were 1:20 in PBS for 11-5F; 1:50 in PBS for anti-dp3; 1:2 in PBS for 33-3D and 1:10 in PBS for 52-3D. PBS was used for all washes. Sections were incubated with the diluted antibody for 60 min at room temperature. After washing (3 × 10 min), sections were incubated with the appropriate antibody conjugate for 60 min at room temperature, washed (3 × 10 min) and mounted.

Conjugated antibodies: fluorescein isothyocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (DAKO Ltd) was diluted 1:50 in PBS and used to detect mouse monoclonal antibodies. FITC-conjugated porcine anti-rabbit immunoglobulin (DAKO Ltd) was used to label the polyclonal antibody to E-cadherin. The conjugate was diluted 1:50 in PBS with 1% BSA and 0·1% Triton-X 100.

Controls were set up by substituting the diluent for the primary antibody, and by substituting PBS for the conjugate.

Triton-X 100 extraction

The aim of this procedure was to remove unattached, soluble, cytoplasmic dp3 from acantholytic cells. Sections were incubated for 30–45 min with 0·5% Triton-X 100 in PBS and washed in PBS before staining for dp3.

Pemphigus antibodies

Sera were diluted 1:10 and 1:40 in PBS. Unfixed sections were incubated with diluted sera for 30 min at 37°C. After washing in PBS (3 × 10 min), sections were incubated for 30 min at 37°C with FITC-rabbit anti-human immunoglobulin (DAKO) diluted 1:50 in PBS, washed (3 × 10 min) and mounted. Controls were set up using serum from a patient with bullous pemphigoid and normal human serum.

Lectin labelling

The optimum working dilutions for PNA and SBA were 100 μg/ml in PBS. Unfixed sections were incubated with the diluted FITC-lectin for 30 min at 37°C, washed (3 × 10 min) and mounted. Control incubations were set up by substituting PBS for the lectin solution, and by preparing solutions in which specific lectin binding to the section was prevented by preincubation of the lectin with the appropriate sugar (D-galactose for PNA and N-acetylgalactosamine for SBA). Inhibited solutions of lectins: one volume of lectin at twice the required concentration was diluted with one volume of 0·3 M saccharide in 0·05 M Tris buffer pH 7·2. The solution was left for 60 min at room temperature before applying it to the sections.

Mounting and microscopy

The stained sections were mounted in Citifluor (Glycerol/PBS solution, Agar Aids, Stanstead, Essex). Sections were examined by epifluorescence and photographed using an Olympus Photomicroscope (model BHS with BH2-RFL attachment).

Results

Immunostaining with antibodies to dp3 and E-cadherin

(a) Normal skin

Anti-dp3 and anti-E-cadherin. There was fine stippled staining of the peripheries of epidermal cells, sparing the bases of the basal cells where they were in contact with the BMZ. The intensity of the stain was weakest between the lateral surfaces of the basal cells, but there was strong staining of the upper poles of the basal cells where they were in contact with suprabasal cells. Uniformly bright staining around the periphery of the cells throughout the rest of the epidermis extended to the granular layer. There was no cytoplasmic staining. When the section was pre-extracted with Triton-X 100 and then incubated with anti-dp3, the peripheral staining around the keratinocytes was occasionally patchily reduced in intensity.

(b) Darier's disease

Anti-dp3. There was a normal peripheral pattern of staining around the keratinocytes in perilesional skin. In the lesional skin there was weak staining around the cells in the 'buds' at the base of the lesion. In some of the acantholytic cells the stain was concentrated within the cytoplasm in a thick, homogeneous rim, while in others the entire cell was diffusely stained. It was possible to discern a granular element to this staining when the focusing was adjusted. There was bright stain outlining, or within, the corps ronds in the granular layer, but no staining in the keratotic plug (Fig. 1). There was a slight

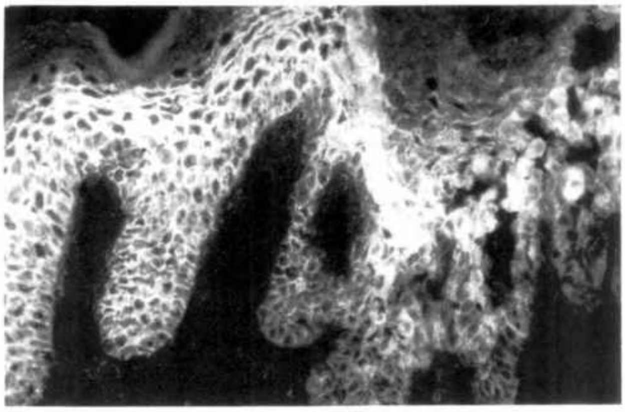

Figure 1. Darier's disease: dp3 is detected in a normal distribution at the periphery of the keratinocytes in perilesional skin, but in the acantholytic cells in lesional skin there is an intense thick rim of fluorescence, or bright diffuse fluorescence (× 100).

decrease in the intensity of the cytoplasmic staining of the acantholytic cells when the section was extracted with Triton-X 100.

Anti-E-cadherin. There was normal labelling of the periphery of the keratinocytes in perilesional skin. In areas of acantholysis there was loss of peripheral staining around many of the acantholytic cells (Fig. 2).

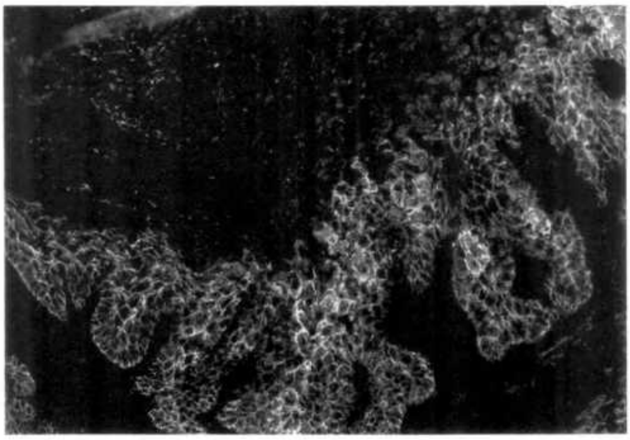

Figure 2. Darier's disease: E-cadherin is detected at the periphery of keratinocytes in perilesional skin, but within the lesion staining is weak or absent around acantholytic cells (× 150).

(c) Hailey–Hailey disease

Anti-dp3. In the perilesional skin there was a normal pattern of peripheral fluorescence around the epidermal cells. In lesional skin the pattern differed from that seen in Darier's disease (Table 2). Although there was diffuse staining of many cells, in 7/9 biopsies bright focal clumps of intracellular fluorescence were observed within the acantholytic keratinocytes (Fig. 3) and within the relatively cohesive cells in the blister roof. In two biopsies, it was not possible to detect focal spots of stain, and diffuse staining predominated. Focal cytoplasmic staining was not diminished by extracting the sections with Triton-X 100.

Material was available from more than one affected individual in three unrelated families (seven biopsies). Clumps of intracellular fluorescence were observed in the acantholytic cells in biopsies from at least one member of each kindred (Table 2).

Sections were incubated with antibodies to dp1/2, dg1 or dg2/3 to compare the patterns of fluorescence with those obtained with anti-dp3 (Table 2). Focal cytoplasmic fluorescence was not detected within the acantholytic cells in the sections stained with anti-dp1/2. Fluorescence was detected in a diffuse pattern or a peripheral ring (Fig. 4). The patterns of stain obtained with anti-dg1 and anti-dg2/3 were similar to those obtained with anti-dp3. Anti dg2/3 produced bright clumps of fluorescence in most acantholytic cells (Fig. 5).

Anti-dp3 had stained 2 sections diffusely. In these sections it was possible to detect some focal staining for dg1 or dg2/3 within acantholytic cells, but diffuse staining predominated (Table 2).

Anti-E cadherin. There was normal labelling of the periphery of the keratinocytes in perilesional skin. In areas of acantholysis the pattern of staining was similar

Table 2. Localization of desmosomal components within the acantholytic cells in biopsies from members of three families (B, U and G) with Hailey–Hailey disease

Family/Patient	Anti-dp3	Anti-dg1	Anti-dg2/3	Anti-dp1/2
B/1	Diffuse	Weak diffuse	Occasional focal	Diffuse
B/2	Focal	Focal	Focal	Diffuse
U/1	Diffuse	Weak diffuse	Occasional focal	Diffuse
U/2	Focal	Focal	Focal	Diffuse
G/1	Focal	Weak diffuse	Focal	Diffuse
G/2	Focal	Focal	Focal	Diffuse
G/3	Focal	Focal	Focal	Diffuse

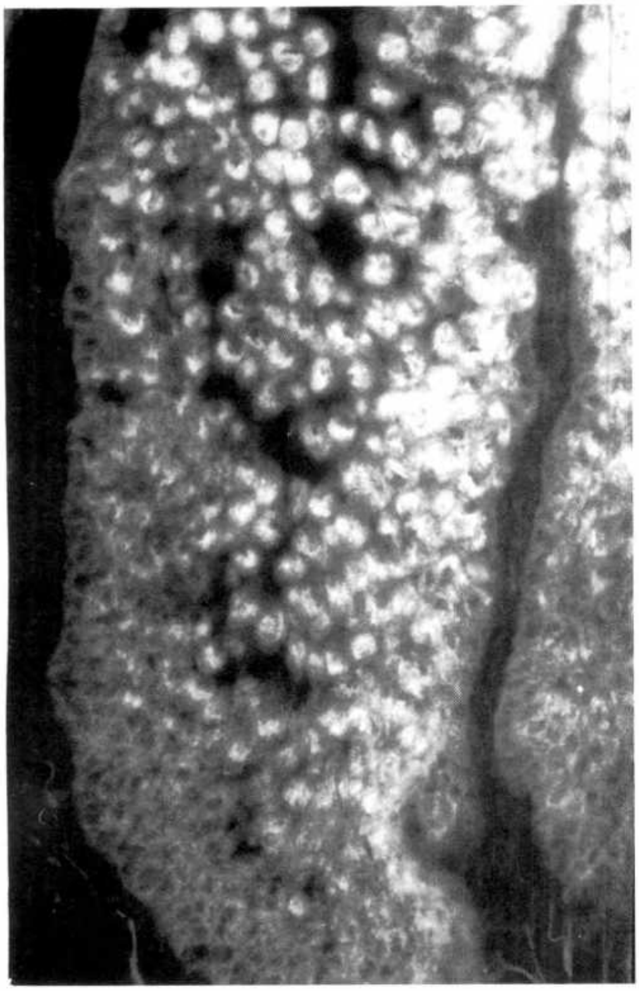

Figure 3. Hailey–Hailey disease: in this section incubated with an antibody to dp3, there is normal expression of dp3 at the periphery of the cohesive keratinocytes, but bright clumps of intracellular fluorescence are present within many acantholytic cells. Some of the acantholytic cells are stained more diffusely (× 200).

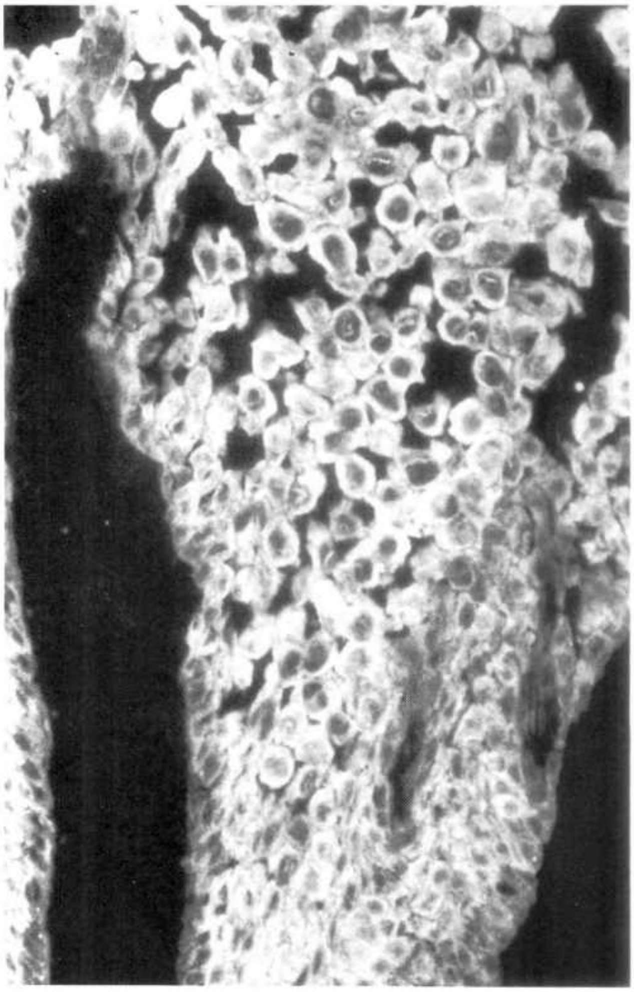

Figure 4. Hailey–Hailey disease: the acantholytic cells are stained diffusely or with a thick peripheral rim when the skin is incubated with an antibody to dp1/2. It was possible to discern a fine granular element to the fluorescence by adjusting the focusing. Anti-dp1/2 never produced the clumps of intracellular fluorescence which were seen with anti-dp3 (× 200).

to that seen in Darier's disease, with loss of peripheral staining around some of the acantholytic cells, but no cytoplasmic staining (Fig. 6).

Lectin labelling

(a) Normal skin

PNA. Staining was either very weak or absent in the basal layer, but increased in intensity in the suprabasal layers. There was peripheral staining around the epidermal cells extending from the suprabasal layer into the granular layer. The stratum corneum was not stained. The base of the basal cells was negative, but in some sections there was a homogeneous band in the BMZ. Staining was inhibited by preincubation of the lectin in 0·3 M galactose.

SBA. Staining was extremely faint or absent in the basal layer but increased in intensity in suprabasal layers, and was maximal in the granular layer. There was peripheral staining around the keratinocytes in the suprabasal layers of the epidermis. The stratum corneum was not stained, and the base of the basal cells was negative, but in some sections there was very faint homogeneous staining in the BMZ. There was also bright granular fluorescence around the dermal vessels. SBA binding was inhibited by pre-incubating the lectin with 0·3 M N-acetylgalactosamine.

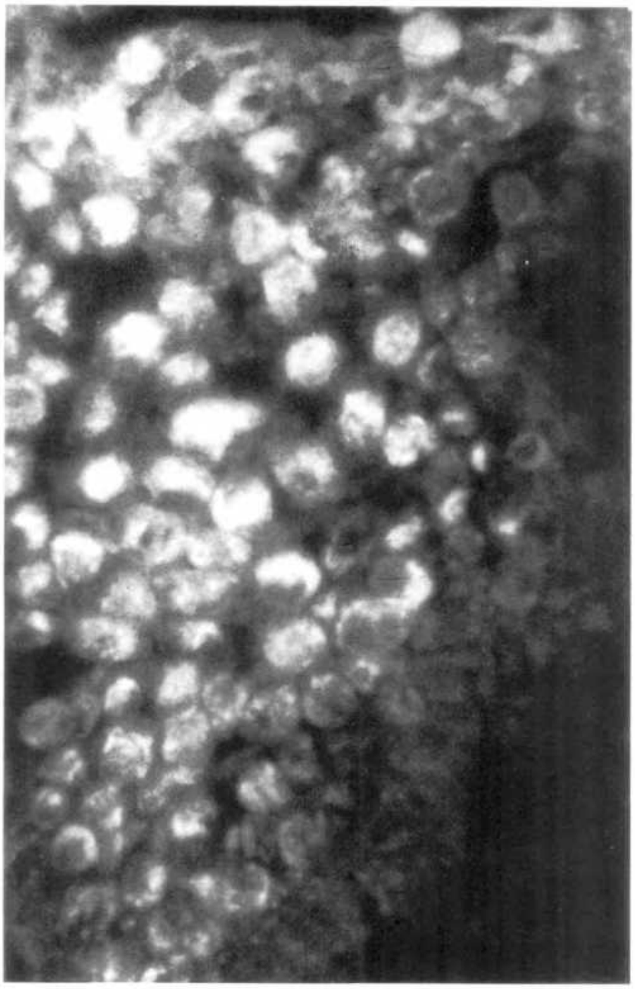

Figure 5. Hailey-Hailey disease: focal intracellular fluorescence is present within many acantholytic cells in this section incubated with anti-dg2/3. The pattern of staining appears similar to that obtained with anti-dp 3(× 400).

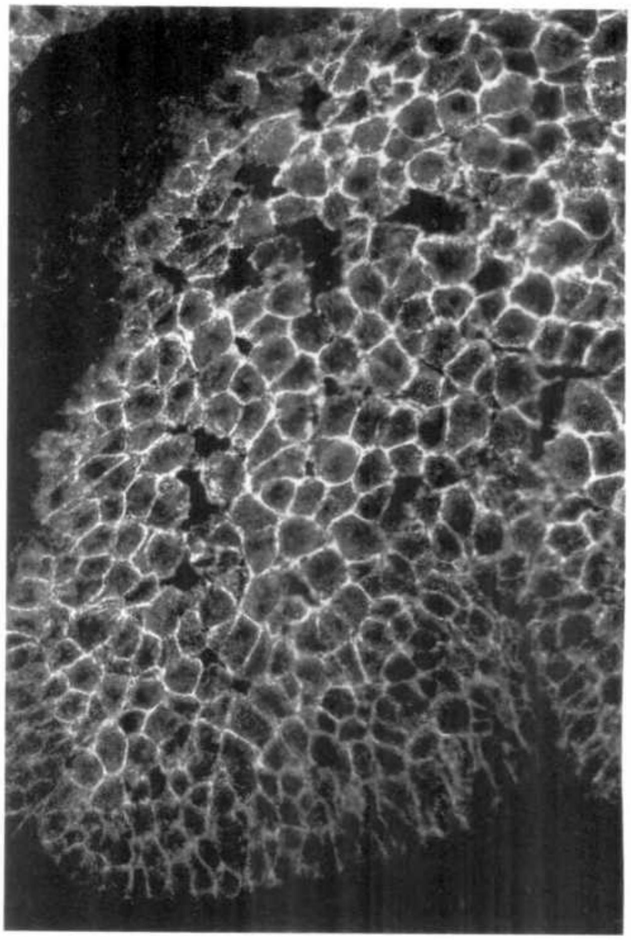

Figure 6. Hailey–Hailey disease: E-cadherin is detected at the periphery of keratinocytes in perilesional skin but fluorescence is weak or absent when there is loss of contact between the cells (× 250).

(b) Darier's disease and Hailey–Hailey disease

PNA. There was a normal pattern of staining in the perilesional skin in both diseases. Staining was absent or very weak around cells in the basal buds below the lesions in Darier's disease. The intensity of labelling was sometimes reduced in areas of acantholysis (Figs 7 and 8). The staining was inhibited by pre-incubation of the lectin in 0·3 M galactose.

SBA. In both diseases the periphery of the keratinocytes in perilesional skin was labelled, but in Darier's disease staining was absent or very weak in the basal buds below the lesion. The periphery of acantholytic cells in both diseases was weakly labelled. Diffuse fluorescence was

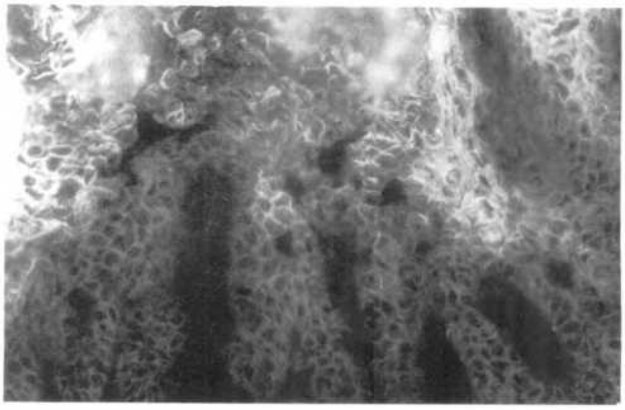

Figure 7. Darier's disease: PNA labels the periphery of keratinocytes in perilesional skin, but the labelling is sometimes rather weak within the acantholytic lesions and the basal buds below the lesion. There is no cytoplasmic fluorescence (× 200).

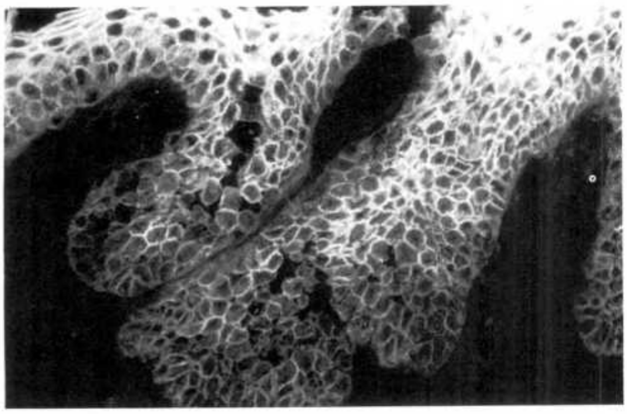

Figure 8. Hailey–Hailey disease: PNA labelling is weak at the periphery of some acantholytic cells but there is normal labelling in perilesional skin (× 200).

detected occasionally. However, in contrast with the results obtained with the desmosomal antibodies, the fluorescence was weak. SBA binding was inhibited by preincubating the lectin with 0·3 M N-acetylgalactosamine.

Pemphigus antibodies

(a) Normal skin

PV and PF sera bound to the cell surfaces of keratinocytes within the epidermis. It was not possible to distinguish between the patterns of PV and PF staining.

BP serum bound to the BMZ, and there was no specific fluorescence using normal human sera.

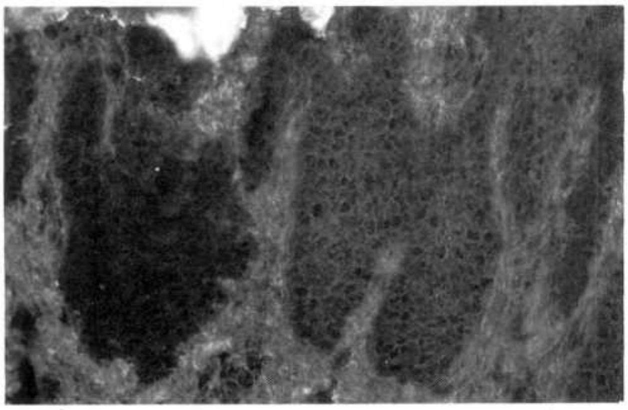

Figure 9. Darier's disease. Immunostaining with PF serum. The pemphigus antibodies label the periphery of keratinocytes in perilesional skin, but there is no fluorescence between the keratinocytes within the lesion. The results were similar in Hailey–Hailey disease (× 200).

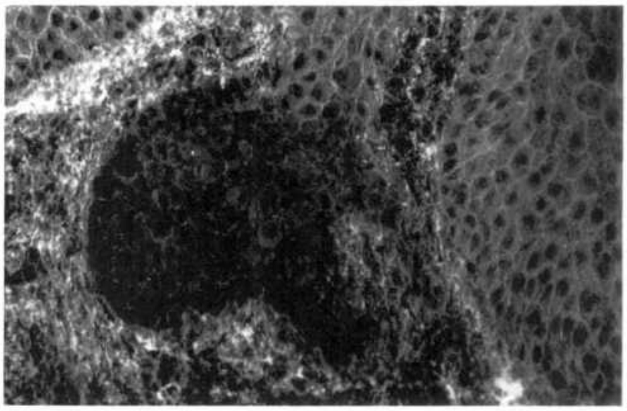

Figure 10. Hailey–Hailey disease. Immunostaining with PV serum. The pemphigus antibodies label the periphery of keratinocytes in perilesional skin, but there is no fluorescence within the lesion. Similar results were obtained in Darier's disease (× 200).

(b) Darier's disease and Hailey–Hailey disease

There was normal intercellular expression of the PV and PF antigens in perilesional epidermis, but reduced or absent fluorescence around the acantholytic cells within lesional skin (Figs 9 and 10). BP serum bound to the BMZ in perilesional and lesional skin, but there was no epidermal fluorescence. There was no fluorescence within the epidermis when sections were incubated with normal human serum.

Discussion

These investigations have shown that dp3 and E-cadherin are present in the epidermis in Darier's disease and Hailey–Hailey disease; that the distribution of internalized dp3 differs in Darier's disease and Hailey–Hailey disease; and that some glycosylated extracellular domains are no longer detected in the acantholytic cells.

Dp3, a plaque protein which is common to desmosomes and adherens junctions, may influence adhesion by regulating the function of transmembrane adhesion molecules.[10–12] Anti-dp3 produced clumps of fluorescence in most acantholytic cells in Hailey–Hailey disease, but the staining in Darier's disease was diffuse. The desmosomal glycoproteins were distributed in a similar clumped pattern within the acantholytic cells of Hailey–Hailey disease.[13] The release of intercellular contacts in cultured epithelial cells results in the endocytotic uptake of desmosomal glycoproteins in membrane vesicles which remain associated with plaque components. Cytoplasmic dots of stain are found particularly in the juxtanuclear region.[20–22] Our observations suggest that dp3 is internalized in the acantholytic cells in a similar distribution to dg2/3 or dg1. This result supports the hypothesis that the cytoplasmic domains of desmosomal glycoproteins link with dp3.[12] Focal staining was not removed by exposing sections to the detergent, Triton-X 100, suggesting that the desmosomal material may be attached to the cytoskeleton. Previous investigators have stated that the immunolocalizations of dp1/2 and dg1 in Hailey–Hailey disease were 'almost the same as that in normal epidermis'. Antibodies to dg2/3 and dp3 were not used in these studies,[23] but our results show these antibodies produce the most obvious clumps of cytoplasmic fluorescence within the acantholytic cells. Dp1/2 staining, in contrast with dp3, was always distributed diffusely in the cytoplasm of acantholytic cells, although dp 1/2 positive aggregates are associated with endocytosed glycoproteins in normal keratinocytes grown in low calcium concentrations.[21]

Focal perinuclear expression of desmosomal components was not apparent in all the biopsies, nor in all the acantholytic cells in a lesion. In cultured keratinocytes focal internalized domains of desmosomal material, formed as a result of trypsinisation or calcium depletion, are cleared after long periods in culture, and staining becomes diffuse.[21] The focal accumulations are probably replaced by more diffuse staining when endocytosed material is degraded in older acantholytic cells. Focal staining for dp3 was not observed in the acantholytic keratinocytes in Darier's disease, but the diffuse pattern of stain was similar to that previously obtained with anti-dp1/2.[13] Desmosomal glycoproteins are also generally distributed diffusely throughout the acantholytic cells in Darier's disease.[13] The lack of focal staining in Darier's disease may reflect the age of the acantholytic cells, with breakdown of internalized material. Lesions of Darier's disease are chronic and generally slow to evolve, while those of Hailey–Hailey disease may appear in a matter of hours. Alternatively, there may be differences in the process of internalization of desmosomal components in the two diseases.

Support for the distinct identities of the diseases has been provided by *in vivo* suction blister studies,[24,25] and the clinical observation that minor trauma induces lesions in patients with Hailey–Hailey disease but not in Darier's disease.[26,27] An abnormality in the adhesion complex between dp3 and desmosomal glycoproteins may be responsible for skin fragility in Hailey–Hailey disease.

E-cadherin can be detected in perilesional skin in both Darier's disease and Hailey–Hailey disease. Staining was decreased at the periphery of some acantholytic cells, but there was no cytoplasmic staining. Membrane domains of E-cadherin are endocytosed when bovine kidney epithelial (MDBK) cells are dissociated during calcium depletion.[22] The antibody may be interacting with an extracellular epitope which undergoes conformational changes when internalized. The pattern of staining obtained with the lectins, PNA and SBA, was similar in clinically normal skin to that seen with the monoclonal antibodies to dgs, but labelling of the acantholytic cells was sometimes reduced, suggesting that extracellular glycosylated portions of adhesion molecules may be lost after acantholysis. Binding of the lectin concanavalin A is also absent or markedly reduced in lesions in both diseases.[28] PV and PF antigens were expressed in perilesional skin, but were no longer expressed by the acantholytic cells. This observation confirms previous reports.[28] Reduced expression of extracellular glycoproteins could arise from conformational changes or internalization of extracellular domains, but loss of these domains may be secondary to proteolysis. Plasminogen activation has been implicated in the pathogenesis of acantholysis in the autoimmune disease pemphigus.[29] Plasminogen has been detected in association with the acantholytic cells in skin biopsies and cultured cells from Darier's disease and Hailey–Hailey disease,[30,31] and the plasminogen activator, urokinase (uPA) is expressed within the acantholytic cells in culture.[31] The changes in shape induced by loss of attachments may be associated with an increased capacity to generate plasmin.[32,33]

Hailey–Hailey disease, in contrast with Darier's disease, can be suppressed by prompt treatment with a moderately potent topical corticosteroid.[26,27] Corticosteroids also inhibit acantholysis in cultures of keratinocytes from Hailey–Hailey disease and may exert their therapeutic effect by inhibiting proteinases.[34] Other factors must contribute to the acantholysis and dyskeratosis in Darier's disease.

Genetically determined mutations in structural proteins may predispose to acantholysis in these dominantly inherited diseases. Even a single amino acid substitution might interfere with adhesion function.[35] In Hailey–Hailey disease, the skin is fragile and abnormal adhesion is immediately revealed by minor trauma. Deficient adhesion in Hailey–Hailey disease may be associated with abnormalities in the complex between desmosomal glycoproteins and dp3. Lesions of Darier's disease are slow to develop and intercellular adhesion is not disrupted by minor trauma. The abnormality may lie within a cytoplasmic component of the adhesion junction or the cytoskeleton, rather than in the extracellular portion of a transmembrane adhesion molecule. The mutation may influence the interactions between the keratin filaments and the desmosomal plaque, causing subtle changes in the stability of the adhesion junctions.

Acknowledgments

This work was supported by the Skin Disease Research Fund and the Oxford District Research Fund.

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British Journal of Dermatology (1992) 127, 344–351.

Increased sensory neuropeptides in nodular prurigo: a quantitative immunohistochemical analysis

F.ABADÍA MOLINA, N.P.BURROWS,* R.RUSSELL JONES,* G.TERENGHI AND J.M.POLAK

Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K
**Department of Dermatology, Ealing Hospital, Middlesex UB1 3HW, U.K.*

Accepted for publication 28 April 1992

Summary

Seven patients with nodular prurigo, five patients with lichenified eczema and seven control volunteers were studied immunohistochemically using antisera to the pan-neuronal marker protein gene product 9.5 (PGP), and the neuropeptides calcitonin gene-related peptide (CGRP), substance P (SP), tyrosine hydroxylase (TH), vasoactive intestinal polypeptide (VIP) and the C-flanking region of neuropeptide Y (C-PON). PGP-, CGRP- and SP-immunoreactivities were also evaluated using image analysis quantification, and the data compared by statistical analysis. No significant changes were noted in the lichenified skin of patients with chronic eczema, compared with the control groups. In contrast, a significant increase in PGP immunoreactive nerve fibres was seen in lesional skin of all nodular prurigo cases studied, when compared with non-lesional skin from the same patient or from control subjects ($P<0{\cdot}001$). In one case massive neural hyperplasia was also identified. Staining for CGRP and SP showed a large increase of immunoreactive nerves in lesional skin of nodular prurigo patients, which closely paralleled that of PGP. Staining with VIP, C-PON and TH was similar in both lesional and non-lesional skin. These results indicate that neural changes in nodular prurigo are associated with an increase of sensory neuropeptides, which could be related to the intense pruritus which accompanies nodular prurigo. The absence of significant changes in lichenified skin suggests that the increase in CGRP- and SP-immunoreactive nerve fibres is a characteristic feature of nodular prurigo and may be important in its pathogenesis.

In 1880 Hardaway[1] described a chronic, pruritic nodular skin disease, and later the same disorder was called prurigo nodularis by Hyde.[2] Patients with this eruption exhibit persistent and intensely pruritic nodules, mainly located on the extensor surfaces of the limbs. Some patients have an associated inflammatory skin disease or a history of chronic insect bite reactions.[3] In other cases, metabolic factors such as iron deficiency or thyroid dysfunction may be important. Psychosocial factors have also been implicated, but in many cases the aetiology remains unknown.[3]

Histologically the condition is characterized by irregular acanthosis with marked hyperkeratosis. Epidermal proliferation may be massive, and the presence of squamoid islands within the epidermis can resemble a well-differentiated neoplasm. However, there is usually continuity between the islands and the overlying epidermis. In addition, there is a stromal reaction with proliferation of fibroblasts and a sparse perivascular infiltrate. In lichen simplex the histological changes may be similar, but are less marked, and clinically areas of lichenification can be distinguished from the discrete nodules of nodular prurigo.

Neurohistological changes in nodular prurigo were first noticed by Johnston,[4] and this report was followed by several others.[5–11] Much emphasis has been placed on neural hyperplasia, a feature first described by Pautrier[5] in 1934 under the title 'L'hyperplasia colossale du tissu nerveaux' and subsequently known as 'Pautrier neuroma'. Recently Lindley and Paine,[11] noted that prominent dermal nerve bundles, or massive neural hyperplasia, were present in only five of the 26 cases they studied, showing that this feature is not a prerequisite for the diagnosis of nodular prurigo. A report by Vaalasti *et al.*[10] showed a generalized increase in CGRP and SP-immunoreactive nerve fibres in some nodular prurigo cases, but the characterization of these nerve fibres was

Correspondence: Dr G.Terenghi, Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K.

not correlated with the presence of neural hyperplasia.

The aim of this study was to elucidate whether neural changes are a specific feature of nodular prurigo, or simply occur as a result of lichenification. Lesional and non-lesional skin from patients with lichen simplex, and control skin from healthy volunteers, were included for comparison.

We hypothesize that there is an increase in nerve fibres in lesions of nodular prurigo, which are likely to be sensory in nature. An increase of sensory neuropeptides might therefore offer an explanation for the intense itching experienced by patients suffering from nodular prurigo.

Immunohistochemical characterization of the nerve fibres was carried out to answer the questions: can neural hyperplasia be visualized by the use of a general neuronal marker, and does the increase selectively involve sensory nerve endings?

Methods

A total of seven patients with nodular prurigo (two men and five women, age range 24–66 years), five patients with lichen simplex (four men and one woman, age range 30–76 years) and seven healthy volunteers with no history of skin disease (four men and three women, age range 24–70 years), were investigated. Elliptical skin biopsies were taken using 2% lignocaine as local anaesthetic. For the lesional skin biopsies the anaesthetic was injected into the peripheral area surrounding the lesion. Two biopsies were taken from each patient with nodular prurigo and lichen simplex, one from lesional skin and the other from a non-lesional area on the contralateral side. Biopsies from all groups were site-matched and taken from the extremities. The skin biopsies were immediately fixed by immersion in Zamboni's fluid for 6 h at room temperature, then transferred into a 0·1 M phosphate-buffered saline (PBS, pH7·2) containing 15% sucrose and 0·1% sodium azide. Frozen-sections (10 μm) were cut, collected on to poly-L-lysine-coated glass slides, and left to dry at room temperature for 1 h before immunostaining.

Immunohistochemistry was carried out according to a modification of the indirect immunofluorescence method.[12] Prior to immunostaining the sections were counterstained with 0·05% pontamine sky-blue, to decrease autofluorescence and to aid location of the immunoreactivity (IR) in the different histological areas evaluated. The areas evaluated were: epidermis, papillary dermis, reticular dermis, blood vessels, sweat glands, and hair follicles. In addition, sections from each skin sample were routinely stained with haematoxylin and eosin for histological purposes.

A variety of antisera were used to study and characterize skin innervation. Immunostaining for the various subpopulations of cutaneous nerves was carried out using previously characterized antisera against the general neuronal marker protein gene product 9.5 (PGP);[13] autonomic catecholaminergic nerve fibres were identified using antisera to tyrosine hydroxylase (TH) and C-flanking peptide of NPY (C-PON).[14] Immunoreactive sensory nerve fibres were demonstrated using antisera against calcitonin gene-related peptide (CGRP)[15,16] and

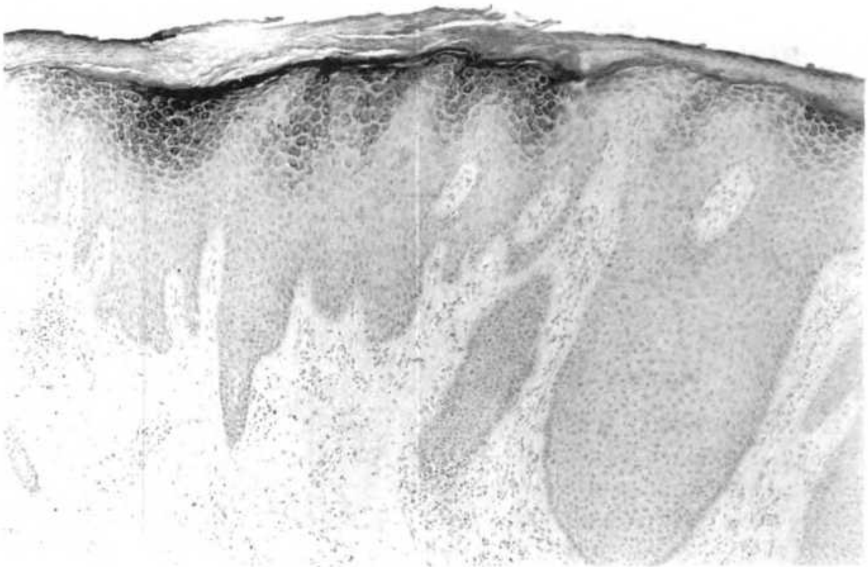

Figure 1. Classical nodular prurigo histopathological features of hyperkeratosis and prominent irregular acanthosis (haematoxylin and eosin × 125).

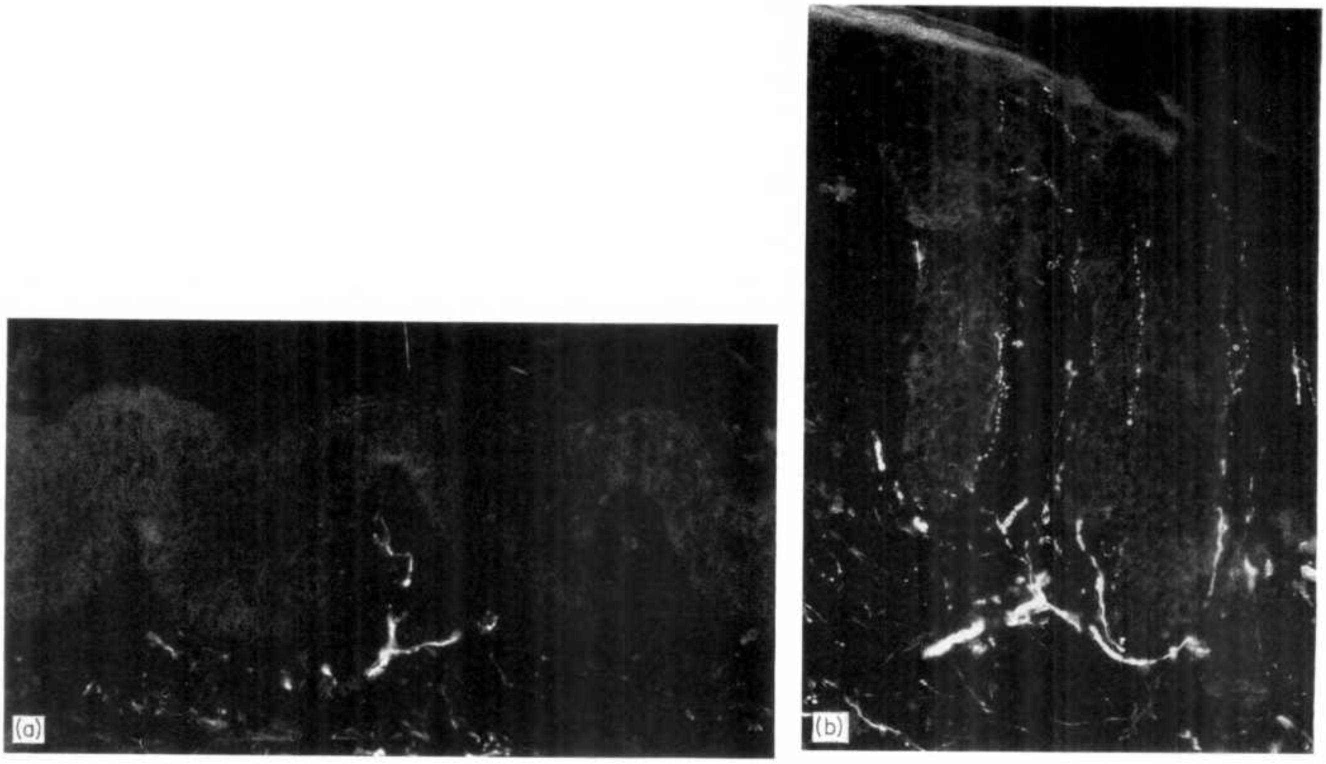

Figure 2. (a) PGP-immunoreactive nerve fibres in unaffected area of skin from a nodular prurigo patient (× 250). (b) PGP-immunoreactive nerve fibres surrounding a dermal papilla and projecting into acanthotic epidermis, from a nodular prurigo patient (× 250).

substance P (SP).[16] The cholinergic innervation was studied using an antiserum to vasoactive intestinal polypeptide (VIP).[17] Sections from each cryostat block were collected sequentially on to several slides so that at least four non-serial sections were stained for the same antigen. The slides were coded and the assessment for light microscopy and image analysis quantification was carried out by independent observers to avoid bias.

Quantitative immunohistochemistry

A low-light video camera (Panasonic WV-1900/C) was mounted on a VANOX fluorescence microscope (Olympus U.K.) and linked to a VIDAS image analyser to assess the total fluorescence area of immunoreactive nerves and intercept counts (a parameter indicative of fibre length) per field. The quantification programme was derived from Terenghi *et al.*[18] Two different areas were considered, and quantified separately: (i) epidermis and papillary dermis; (ii) reticular dermis. Two random sections from each biopsy were analysed for PGP, CGRP and SP immunostaining. Three fields (× 20 objective magnification) were then measured in both areas as defined above. The means and medians of the six measures for a given immunostaining in each area were calculated and compared by statistical analysis using ANOVA and Mann–Whitney *U*-tests.

Results

Examination of haematoxylin and eosin-stained sections confirmed the typical histopathological features of nodular prurigo, with prominent hyperkeratosis and markedly irregular acanthosis (Fig. 1) overlying a lymphohistiocytic infiltrate in the upper dermis. Lichen simplex was characterized by more regular acanthosis, with hyperkeratosis and occasional parakeratosis.

In control samples immunostaining for the general neuronal marker protein gene product 9.5 (PGP), showed an extensive subepidermal network of nerve fibres branching into the dermal papillae and surrounding the vascular loops (Fig. 2a). Some of the fibres appeared to penetrate the epidermis as free nerve end terminals. In the dermis, immunostaining was seen in both single nerve fibres and nerve bundles surrounding dermal blood vessels, sweat glands and hair follicles. In

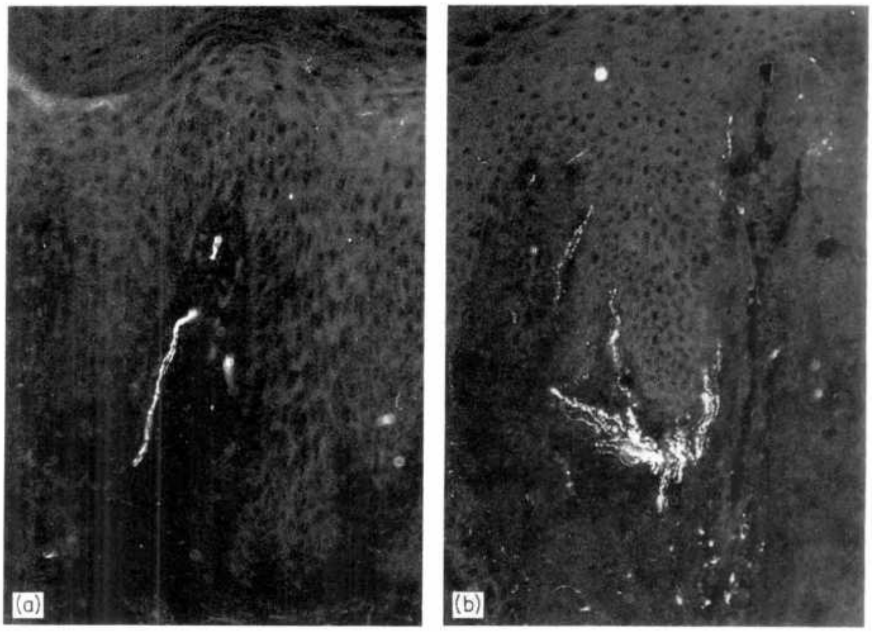

Figure 3. (a) CGRP-immunoreactive nerve fibres in unaffected area of skin from a nodular prurigo patient (× 250). (b) Immunoreactive CGRP nerve fibres from lesional skin of a nodular prurigo patient (× 250).

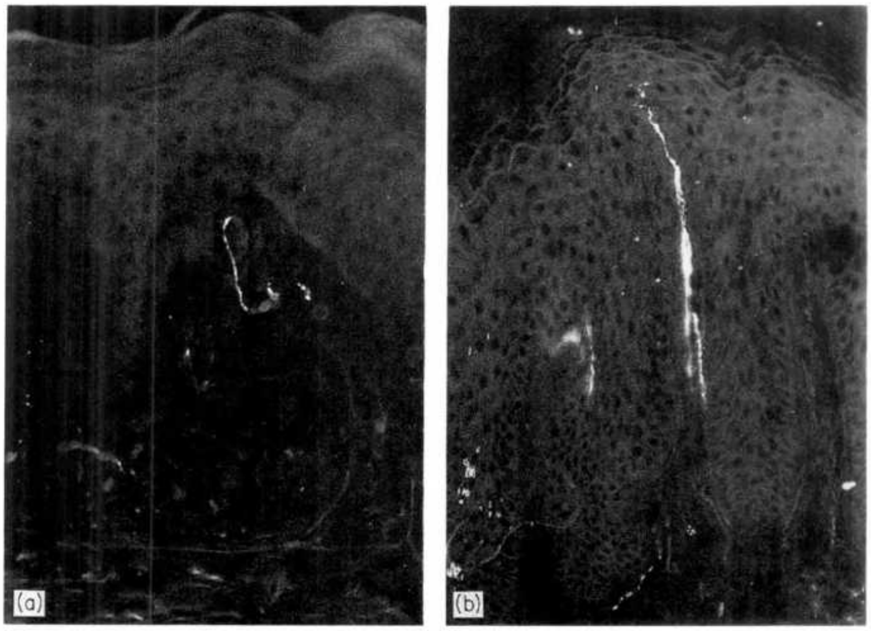

Figure 4. (a) SP-immunoreactive nerve fibre in unaffected area of skin from a nodular prurigo patient (× 250). (b) SP-immunoreactive nerve fibres from lesional skin of a nodular prurigo patient (× 250).

control skin, calcitonin gene-related peptide (CGRP) immunostaining paralleled that of PGP but showed fewer and thinner immunoreactive nerve fibres (Fig. 3a). Immunoreactive substance P (SP) nerve fibres were less abundant and were restricted to the papillary plexus (Fig. 4a), and occasionally in dermal nerve bundles.

The changes seen in nodular prurigo are summarized in Table 1. The lesional biopsies of all nodular prurigo cases exhibited an increase in the number of dermal and epidermal PGP-immunoreactive nerve fibres when compared with non-lesional skin, lichenified skin, or control biopsies. The nerve fibres were particularly abundant in the papillary dermis, and projected into the epidermis (Fig. 2b). No changes were found around sweat glands, blood vessels or hair follicles. One of the patients exhibited the classical features of 'Pautrier neuroma', as defined by the presence of grossly enlarged PGP immunoreactive nerve bundles, some of which extended to

	Epidermis	Papillary dermis	Reticular dermis	Sweat glands	Hair follicles	Dermal blood vessels
PGP	↑	↑	↑	←→	←→	←→
CGRP	←→	↑	↑	←→	←→	←→
SP	←→	↑	↑	NA	←→	NA
VIP	NA	NA	←→	←→	←→	←→
C-PON	NA	NA	NA	NA	NA	←→
TH	NA	NA	NA	←→	NA	←→

Table 1. Semiquantitative analysis of immunostaining in lesional biopsies of nodular prurigo. ↑ = increase, ←→ = no changes. NA = not applicable

Abbreviations: PGP, protein gene product; CGRP, calcitonin gene-related peptide; SP, substance-P; VIP, vasoactive intestinal polypeptide; C-PON, C-flanking region of NPY; TH, tyrosine hydroxylase.

the dermal papillae (Fig. 5a). These abnormally large nerve bundles were also visible in the haematoxylin and eosin stained sections and 'schwannoma' formation could be identified.[9,19–21]

When lesional skin from nodular prurigo patients was compared with the rest of the groups studied, immunostaining for CGRP and SP revealed changes similar to those found when using PGP antiserum. Prominent increases were noticed for both CGRP and SP-containing nerve fibres, which were located mainly in the epidermal and subepidermal layers (Figs 3b and 4b). In the case with massive neural hyperplasia, there were large CGRP-immunoreactive nerve bundles, as found with PGP immunostaining (Fig. 5b). Neural hyperplasia was not evident with SP immunostaining. In all nodular prurigo cases, the changes of PGP, CGRP and SP immunoreactive nerve fibres were restricted to the nodules of lesional skin. No differences were noticed between any of the groups in the amount of nerve fibres immunoreactive for vasoactive intestinal polypeptide (VIP), C-flanking region of neuropeptide Y (C-PON) and tyrosine hydroxylase (TH).

In order to obtain a more objective assessment of the reported changes above, quantitative immunohistochemical analysis was carried out for PGP-, CGRP- and SP-containing fibres in two areas: (i) epidermis and papillary dermis; (ii) reticular dermis. The patient who exhibited massive neural hyperplasia was excluded from this study, because of the obvious large-scale abnormalities. The fluorescence-immunostained area for PGP,

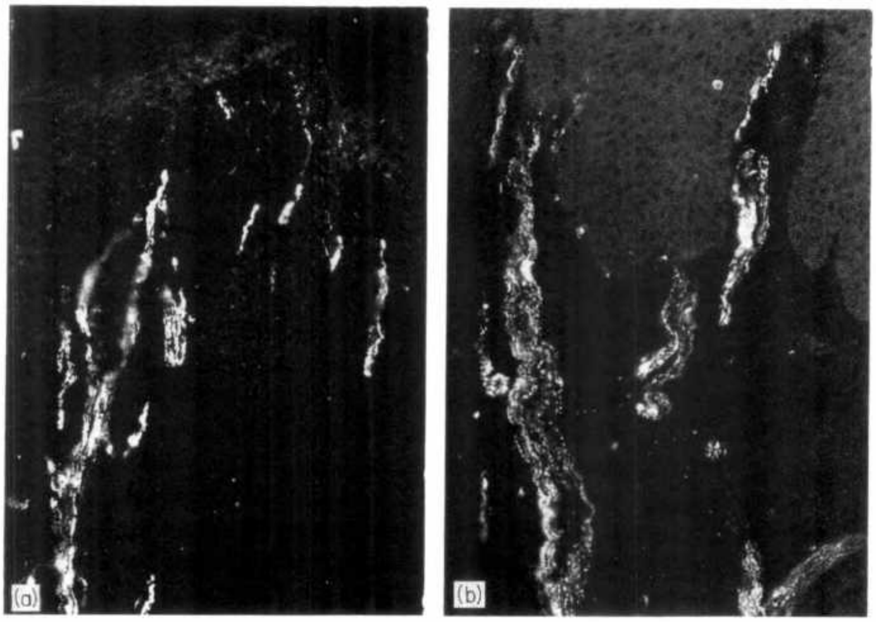

Figure 5. (a) Hyperplastic PGP-immunoreactive nerve fibres beneath the epidermis, in lesional skin of a nodular prurigo patient. Thicker nerve bundles reach up to the dermal papillae (× 250). (b) Neural hyperplasia revealed by CGRP-immunoreactive nerve fibres in lesional skin of a nodular prurigo patient (× 250).

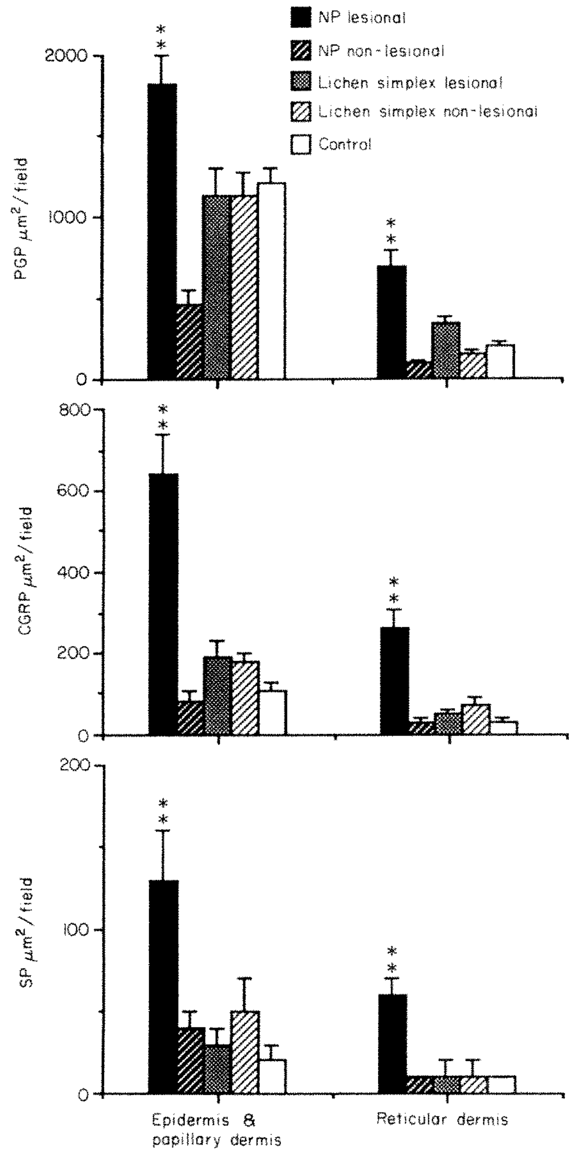

Figure 6. Histograms showing the total field fluorescent area (μm²) of PGP-, CGRP- and SP-immunoreactive nerves, in the different types of skin investigated. The values are expressed as means, with the SEM represented by error bars. $^{**}P<0{\cdot}001$.

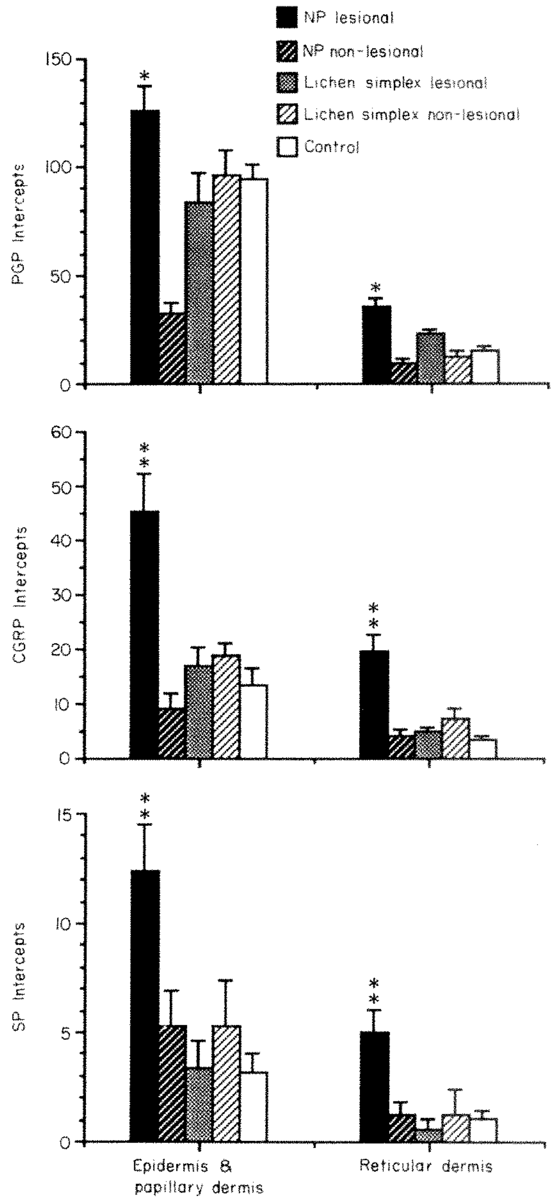

Figure 7. Histograms showing the intercept counts of PGP-, CGRP- and SP-immunoreactive nerves, in the different types of skin investigated. The values are expressed as means, with the SEM represented by error bars. $^{*}P=0{\cdot}05$; $^{**}P<0{\cdot}001$.

CGRP or SP immunoreactive nerve fibres (Fig. 6) was increased significantly in epidermis/papillary dermis and in reticular dermis of nodular prurigo lesional biopsies ($P<0{\cdot}001$) when compared with staining in the other four groups. Intercept counts for PGP immunostaining, were also significantly increased in epidermis/papillary dermis and reticular dermis of nodular prurigo lesional biopsies, ($P<0{\cdot}05$) when compared with the other samples (Fig. 7).

Intercept counts of CGRP and SP immunoreactive nerves in epidermis/papillary dermis and reticular dermis of nodular prurigo patients showed a highly significant increase ($P<0{\cdot}001$) compared with the other groups (Fig. 7).

Discussion

In this study we have shown a proliferation of PGP immunoreactive nerve fibres in the nodules of nodular prurigo, which were characterized to be mostly sensory nerves by positive staining for CGRP and SP. In the only case of massive neural hyperplasia, as defined by PGP immunostaining, the majority of nerves within the nerve bundles were also identified as sensory, and immunoreactive for CGRP. These findings are in agreement with those of Lindley and Payne,[11] who also identified massive neural hyperplasia in less than 20% of nodular prurigo cases.

CGRP and SP neuropeptides are known to be present in the afferent sensory pathways.[22–27] CGRP immunoreactive nerves constitute one of the largest subpopulations of peptidergic primary sensory neurons in the skin, and the concomitant increase of CGRP and SP immunoreactive fibres is to be expected, as all the primary afferent neurons containing SP also co-store CGRP.[24]

Quantitative analysis confirmed that the increase of immunoreactive nerves in nodular prurigo was statistically significant, as shown with both measured parameters. The changes were found in both epidermis/papillary dermis and reticular dermis, although in the former area the neural proliferation was more evident. Interestingly, the difference between nodular prurigo and control tissues was more evident with immunostained area measurements than intercept counts, particularly for PGP-immunoreactive nerves. This is most probably due to the fact that there was a numerical increase in both single fibres and nerve bundles, the latter contributing more to area values than nerve fibre length as defined by intercept counts.

Traditionally, the intense pruritus in nodular prurigo has been related to neural hyperplasia,[9] although nowadays there are conflicting views, since it has been established that this feature is not invariably present in nodular prurigo cases.[3–11]

The demonstrated sensory nature of the abnormally increased subpopulation of nerve fibres, exhibited in all the nodular prurigo cases studied, might provide a clue to the understanding of this skin condition. Indeed CGRP and SP exhibit roles other than in sensory processing. Both peptides have been reported to be potent histamine-releasing factors,[28–31] a fact that might offer an explanation for the intense itching experienced by nodular prurigo patients. It is also established that SP is a mediator in neurogenic inflammation[32] and that CGRP potentiates the plasma extravasation induced by SP.[33] The prominent role of sensory neuropeptides in nodular prurigo is also consistent with the lack of increase of adrenergic or cholinergic nerve subpopulations, as shown by immunostaining for TH, C-PON and VIP.

Interestingly, SP has been found to act as a trophic factor, enhancing the growth of fibroblasts[34] and keratinocytes.[35] These properties may be relevant in the pathogenesis of the disease, in view of the acanthosis and fibroblast proliferation characteristic of nodular prurigo.

It is a matter of speculation whether these changes are causative in, or a consequence of, nodular prurigo. It could be argued that if neural proliferation was secondary to trauma and scratching, similar findings should be observed in lichenified skin. However, no changes of sensory neuropeptide could be found in skin biopsies from patients with lichen simplex, a fact which may be of use in differentiating between the two conditions. Furthermore, the increase of sensory nerves was restricted to the nodules, suggesting that a focal increase of CGRP- and SP-immunoreactive nerves might play an important role in the development of the pruritic nodules.

Acknowledgments

Dr F.Abadia is in receipt of a grant of the 'Programa Nacional de becas de formación de personal investigador en el extranjero' from the 'Ministerio de Educación y Ciencia' of Spain. The authors are grateful to the Colt Foundation, the BUPA Medical Foundation and the Freemasons' Grand Charity for generous financial support.

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British Journal of Dermatology (1992) 127, 352–358.

Similarity between mycobacterial and human epidermal antigens

TH.W.VAN DEN AKKER,*† B.NAAFS,* A.H.J.KOLK,‡
E.DE GLOPPER-VAN DER VEER,† R.A.M.CHIN, A LIEN* AND TH.VAN JOOST*
*Departments of *Dermato-Venereology and †Immunology, Academic Hospital Dijkzigt, Rotterdam, The Netherlands*
‡N H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam, The Netherlands

Accepted for publication 29 April 1992

Summary

Eight out of 17 mouse anti-*Mycobacterium leprae* monoclonal antibodies (MAb) were previously observed to react with human nerve and skin antigenic determinants in cryostat sections, using an indirect immunoperoxidase technique. These observations suggested that antigenic mimicry may be involved in the development of the clinical manifestations of leprosy. In the present study we have extended our earlier findings by investigating sera from leprosy patients and MAb using Western blot technique. It was observed that 30 sera and their corresponding $F(ab')_2$ fragments from isolated IgG fractions of both tuberculoid and lepromatous patients reacted with 40–50 epidermal proteins of molecular weights (MW) ranging from 10 to 130 kDa. Sera from 14 controls, however, showed similar reactivity patterns. Absorption of nine patient and control sera with *M. tuberculosis, M. marinum* and *M. kansasii* resulted in the removal of several components of different MW in nine, four and three cases, respectively. No consistent differences between sera from leprosy patients and controls were observed. Four out of eight MAb against *M. leprae* which reacted with determinants in human epidermis and/or dermis in skin cryostat sections reacted with epidermal proteins of MW higher than 39 kDa in Western blot. Four MAb which showed reactivity in cryostat sections did not react in Western blot. Another four MAb did react with human epidermal proteins in Western blot but did not react in cryostat sections, indicating that the MAb were reacting with different epitopes in the two systems. Five MAb did not react with human epidermal proteins either in cryostat sections or in Western blot. Because all sera that were investigated contained antibodies against antigenic determinants of epidermal proteins, some of which are shared with *M. leprae* and cultivatable environmental mycobacteria, it is tempting to speculate that antigenic mimicry could be involved in autoimmune skin diseases which are induced and/or maintained by environmental micro-organisms.

Molecular mimicry between host and parasite was first mentioned by Damian in 1964,[1] and indicated the sharing of antigenic determinants by the host and the parasite. The similarity of antigenic determinants between human tissues and environmental micro-organisms was suggested as a possible alternative explanation for the clinical manifestations of leprosy by Naafs *et al.*[2] On the one hand, molecular mimicry enables the parasite to avoid detection and destruction by the host: a state of tolerance. Such a process may occur in lepromatous leprosy, one pole of the leprosy spectrum. In lepromatous leprosy, cell-mediated immunity (CMI) towards *Mycobacterium leprae* antigen is absent, and large numbers of *M. leprae* are found in the tissues. On the other hand, molecular mimicry may induce a state of autoimmunity. Tuberculoid leprosy, the other pole of the leprosy spectrum, serves as an example of such a state. A high CMI towards *M. leprae* without bacilli in the patients' tissues, as observed in tuberculoid leprosy, is accompanied by extensive granuloma formation and tissue destruction in the dermis and the peripheral nerves. This may represent an immunological response to 'self antigens'.

Previously, it was observed that sera from healthy blood donors and patients with various diseases reacted with cytoplasmic epidermal antigens.[3,4] Neural autoantibodies were observed in sera from leprosy patients by Wright *et al.*[5] We have shown previously that mouse anti-*M. leprae* monoclonal antibodies (MAb) reacted

Correspondence: Dr B.Naafs, Department of Dermato-Venereology, Academic Hospital Dijkzigt Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

with human (epi) dermal and peripheral nerve determinants suggesting that 'antigenic mimicry' may be involved in the pathogenesis of leprosy.[2]

In this article we have extended our previous studies in an attempt to characterize further the determinants detected by murine MAb against *M. leprae* and sera from leprosy patients using Western blot technique. Absorption studies with three species of cultivatable mycobacteria were also performed in order to investigate whether other micro-organisms also share antigenic determinants with the human epidermis.

Methods

Human epidermis extract

Human abdominal skin was obtained from a healthy woman who had undergone abdominoplasty in the Department of Plastic Surgery of our hospital. Extracts were prepared according to the method described by Peterson and Wuepper.[6] Briefly, the epidermis was separated from the dermis by immersion of the skin in 2 M NaBr at 37°C for 60 min. The epidermis was subsequently frozen in liquid nitrogen, pulverized and homogenized in 100 ml 0·025 M phosphate-buffered saline (PBS, pH 7·4), containing 2 mM phenylmethylsulphonylfluoride and 8 M urea. The homogenate was then centrifuged at 40,000 *g* for 30 min at 4°C. The supernatant was discarded, and the membrane-rich pellet was resuspended in 1% dimethylsulphoxide in PBS containing 1% sodium dodecyl sulphate (SDS). The protein concentration of the membrane suspension was determined by measuring the absorbance at 280 nm. The protein concentration was determined to be 20·3 mg/ml using bovine serum albumin as a standard.

Sera

Sera from 30 patients with leprosy, classified according to Ridley and Jopling,[7] who were receiving treatment, were used. Paucibacillary patients were treated with rifampicin and dapsone, and multibacillary patients were also treated with clofazimine. Control sera were obtained from patients with a number of other dermatological conditions such as androgenetic alopecia, skin tags and dermatosis papulosa nigra.

Monoclonal antibodies against M. leprae

A panel of 17 mouse monoclonal antibodies (MAb) against *M. leprae* was used. The MAb had been prepared and checked for their reactivity with *M. leprae* antigen in Western blot and ELISA as previously described.[8] The MAb and their specificities are listed in Table 1.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Extracts of human epidermis were subjected to SDS–PAGE according to Laemmli[9] using 11% separating gels with 0·1% SDS. Samples of epidermal protein (100 μg) were treated with 5% 2-mercaptoethanol and 2% SDS and boiled in a water bath at 100°C for 5 min. A mixture of prestained proteins consisting of phosphorylase B (130 kDa), bovine serum albumin (BSA) (75 kDa), ovalbumin (50 kDa), carbonic anhydrase (39 kDa), soybean trypsin inhibitor (27 kDa) and lysozyme (17 kDa) was used as marker (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Western blot

The separated proteins in the gel were electrophoretically blotted on to nitrocellulose membrane using a Bio-Rad transblot cell (Bio-Rad Laboratories). After blotting, nitrocellulose strips were blocked with PBS containing 1% BSA. The nitrocellulose strips were then incubated with sera (dilution 1:250) or MAb (dilution 1:250), washed with PBS containing 0·05% Tween and incubated with (a) goat anti-human Ig alkaline phosphatase conjugate (GAHu/Ig-AP) or (b) goat anti-mouse Ig alkaline phosphatase conjugate (GAM/Ig-AP) both diluted 1:200, respectively (Southern Biotechnology Associates Inc. Birmingham, AL, U.S.A.). Blots were stained with Naphtol AS-MX phosphate (No. N5000, Sigma) and Variamine Blue RT Salt (No. A5254, Sigma, St Louis, MO, U.S.A.) as a substrate for AP.

Absorption studies

Sera were absorbed with 2-mg pellet fractions of sonicates of *M. marinum*, *M. tuberculosis* and *M. kansasii* as described previously.[10]

Results

Sera from leprosy patients and controls

Sera from 30 tuberculoid and lepromatous patients and 14 controls were tested with the extract of human epidermis using Western blot. Representative results from 10 sera from leprosy patients and 10 controls are shown in Figure 1(a) and (b). Forty to fifty epidermal proteins ranging from 10 to 130 kDa reacted with sera from both leprosy patients and controls. Although each patient's serum had its own characteristic reactivity

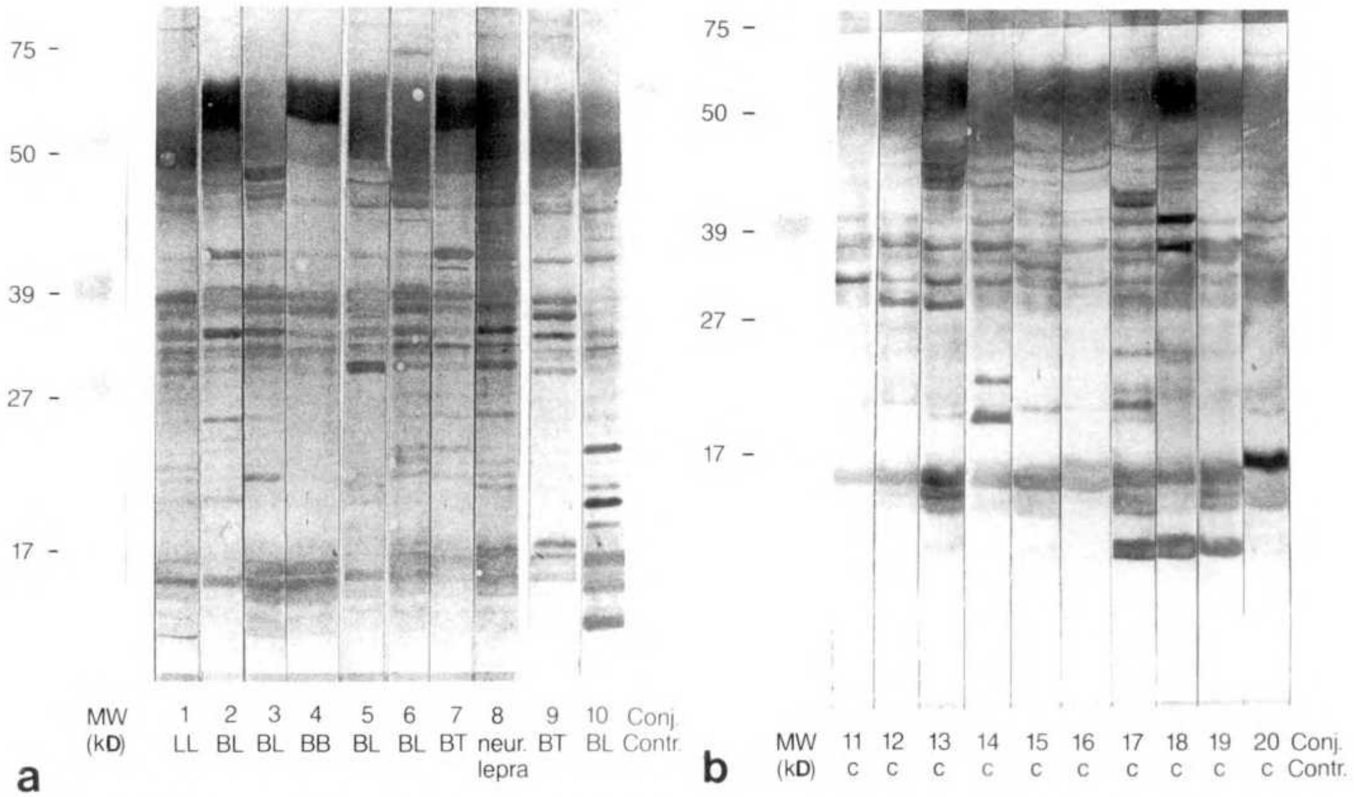

Figure 1. Reactivity of sera from leprosy patients (a) and control sera (b) using Western blot with the extract of human epidermis. This extract was subjected to SDS–PAGE. Proteins in the gel were subsequently electrophoretically blotted on to nitrocellulose membrane. The strips were incubated with sera from leprosy patients (a) and controls (b) followed by incubation with goat anti-human Ig conjugated with alkaline phosphatase.

Table 1. Reactivity of monoclonal antibodies (MAb) against *Mycobacterium leprae* with the extract of human epidermis in Western blot and in cryostat sections of normal human skin using an indirect immunoperoxidase technique

Monoclonal antibody	Isotype	*M. leprae* W. blot reaction*	Epidermis W. blot reaction†	Reactivity on cryosections‡	
				Epidermis	Dermis
F 116-1	IgM	12	40, 42–45, >65	IC	—
F 116-13	IgM	12	40, 48–60	SC, SG, IC, D	—
F 116-14	IgM	12	40, 48–60	SC, SG, IC, D	—
F 116-12	IgG1	18	—	SG	M, N, PV, F
F 116-17	IgG2a	18	—	SC, SG	M, N, A, PV
F 116-5	IgM	26	>43	—	—
F 47-9	IgG1	36	—	—	—
F 116-9	IgG1	36	45	—	—
F 116-4	IgM	30–50	41, 45–65	—	—
F 116-16	IgG	65	45–65	—	—
F 116-19	IgM	65	—	ALL K	PV
F 116-21	IgM	65	40, 47	SG, IC	PV, PNA
F 47-10	IgG1	65	—	—	—
F 67-18	IgG1	65	—	—	—
F 88-1	IgG2a	65	—	BC	M, PV
F 47-1	IgG1	>200	—	—	—
F 47-21	IgG1	Phenolic glycolipid	—	—	—

* Molecular weight (kDa) of *M. leprae* antigens in Western blot.

† Molecular weight (kDa) of epidermis determinants above 39 kDa in Western blot.

‡ Abbreviations: IC, intercellular; SC, stratum corneum; SG, stratum granulosum; D, desmosomes; K, keratinocytes; A, arterioles; PV, perivascular; M, smooth muscles; PNA, peripheral nerve axon; BC, basal cells; N, nerves.

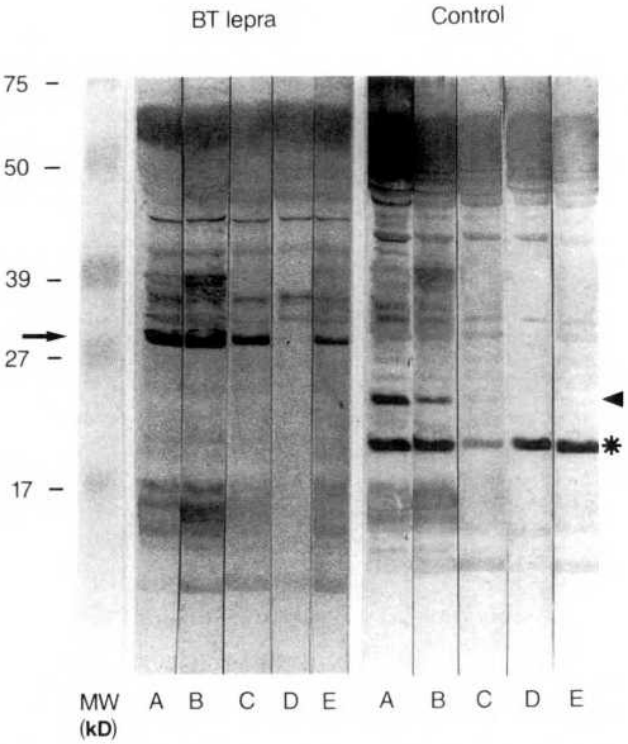

Figure 2. Reactivity of the serum from a BT leprosy patient and the serum from a control individual, with the extract of human epidermis before and after absorption. Lane A, reaction of the unabsorbed sera: lane B, reaction of sera after incubation with phosphate buffer used for absorption; lane C, reaction after absorption with 2-mg pellet of sonicated *M. marinum*; lane D, reaction after absorption with 2-mg pellet of sonicated *M. tuberculosis*; lane E, reaction after absorption with 2-mg pellet of sonicated *M. kansasii*. Note that some bands are absent: lane D 30-kDa band (→) in serum from patient with BT leprosy. Some bands are weak: lane C 20-kDa band (*) in control serum. Some are absent after absorption of the serum with any of the three mycobacterial strains (◀). Some bands are absent after absorption of the serum with only one mycobacterial strain; here *M. tuberculosis*, lane D 30-kDa band, BT lepra serum (→).

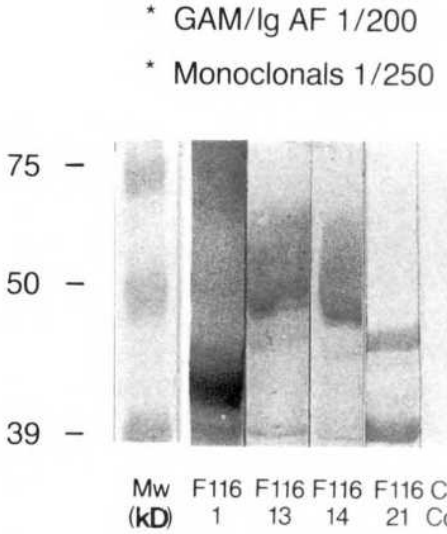

Figure 3. Reactivity of MAb F 116-1, F 116-13, F 116-14 and F 116-21 with the extract of human epidermis using GAM/Ig-AP as a conjugate. Characteristics of these MAb are depicted in Table 1. MAb F 116-1, F 116-13 and F 116-14 are directed against a 12-kDa protein of the *M. leprae* sonicate. A similar staining pattern of the 40-kDa protein and the 48–60 kDa proteins for MAb F116-13 and F 116-14 can be seen. MAb F 116-1, also directed against a 12-kDa *M. leprae* protein, behaves differently and stains the 42–45 kDa and > 65-kDa proteins. F 116-21 directed against a 65-kDa *M. leprae* protein stains the 40-kDa and 47-kDa proteins.

pattern, most pronounced in the 17–30 and 40–45 kDa range, sera from patients and controls reacted with protein bands in the 10–17, 30–40 and 45–65 kDa range.

Absorption studies were performed to investigate which bands were obtained due to antibodies cross-reacting with mycobacterial antigens. Sera from seven leprosy patients and two controls were absorbed with 2-mg pellets of sonicates of *M. marinum*, *M. tuberculosis* or *M.kansasii*. As shown in Figure 2, using absorbed sera some bands could no longer be observed and others were weak. Some bands disappeared after absorption with the sonicates of all three mycobacterial strains, whereas other bands disappeared with one sonicate only (Fig. 2). Of the three mycobacterial strains, *M. tuberculosis* was most effective in absorption of sera from both leprosy patients and controls. Absorption of the nine sera with *M. tuberculosis*, *M. marinum* and *M. kansasii* resulted in the disappearance of several bands of various MW in the blot in nine, four and three cases, respectively.

Monoclonal antibodies against M. leprae

Seventeen MAb against *M. leprae* were tested with the extract of human epidermis using Western blot technique (Table 1). MAb F 116-1, F 116-13 and F 116-14 were known to be directed against a 12-kDa protein of *M. leprae* sonicate.[2] In Western blot, using the extract of human epidermis (Fig. 3) MAb F 116-13 and F 116-14 showed identical reactivity, especially with the 40-kDa protein and with proteins in the 48–60 kDa range, whereas F 116-1 reacted with a 40-kDa protein and with proteins in the 42–45 kDa range, and those above 65 kDa. In the cryostat sections, MAb F 116-13 and F 116-14 also showed similar reactivity with determinants in the stratum corneum, stratum granulosum, intercellular substance and on desmosomes, whereas MAb F 116-1 reacted only with the intercellular substance in the epidermis (Table 1).[2] MAb F 116-21 reacted with a 65-kDa protein of *M. leprae* sonicate.[2] In

Western blot, using the extract of human epidermis (Fig. 3) MAb F 116-21 reacted with proteins of MW 40 and 47 kDa. In cryostat sections the stratum granulosum and intercellular substance of the epidermis and perivascular structures of the dermis were stained by MAb F 116-21, and this MAb also reacted with peripheral nerve axon.[2] MAb F 116-5, F 116-9, F 116-4 and F 116-16 reacted with the extract of human epidermis in Western blot, but not with skin determinants in cryostat sections. MAb F 116-12, F 116-17, F 116-19 and F 88-1 reacted with skin determinants in cryostat sections, but not with the extract of human epidermis in Western blot. MAb F 47-9, F 47-10, F 67-18, F 47-1 and F 47-21 (Table 1) did not react in either Western blot or in cryostat sections of the skin.

Discussion

Following our early studies[2] concerning the reactivity of MAb against *M. leprae* in normal human skin using an indirect immunoperoxidase technique, this present study concerns investigations with the same MAb using Western blot technique. In addition, sera from leprosy patients and controls before and after absorption with sonicates of mycobacteria were investigated. The results of these investigations provide further evidence of the similarity between antigenic determinants of mycobacteria and human epidermis.

It was observed that sera from both leprosy patients and controls reacted with epidermal determinants in Western blot. No consistent differences between the sera from patients with the different forms of leprosy and controls were observed. It was noted that each individual, whether leprosy patient or control, showed a specific banding pattern ('finger-print') probably reflecting the unique history of the individual with respect to previous encounter(s) with micro-organisms. A number of these bands disappeared completely, others partially, after absorption of the sera from the leprosy patients and controls with sonicates of *M. marinum*, *M. tuberculosis* or *M. kansasii*, indicating similarity between mycobacterial and epidermal determinants. Complete disappearance of a protein band indicates that all antibodies in the serum reactive with that particular determinant also reacted with mycobacterial antigens. Partial disappearance could be due to: (a) a low affinity of the cross-reacting antibodies to the mycobacterial antigens; (b) incomplete or partial absorption of particular antibodies with the sonicate; (c) the presence in the serum of antibodies to other antigenic determinants in the protein band which are dissimilar to the mycobacterial antigens; or (d) the presence of antibodies in the serum that react with determinants of a different protein with the same MW as the epidermal antigen detected by the cross-reacting antibodies.

Of these four possibilities, (b) is the least likely to have occurred, because when the amount of mycobacterial sonicate was increased, no additional activity from the sera could be absorbed out, and no additional bands disappeared. It is not clear which of the three remaining possibilities was most relevant in our study. Probably, all three phenomena occurred. The heterogeneity of serum antibodies became further apparent with the observation that some bands disappeared after absorption with sonicates of all three mycobacterial strains, whereas other bands only disappeared after absorption with the sonicate of one strain, e.g. *M. tuberculosis* (Fig. 2). Thus cross-reactivity of the sera appeared to exist both at the level of common, and specific, mycobacterial epitopes.

Because the absorption of all nine sera with *M. tuberculosis* sonicate resulted in the removal of several protein bands in the Western blot using the extract of human epidermis, it appears that many individuals have specific antibodies to *M. tuberculosis* which cross-react with determinants in the epidermis. The primary sensitization in our leprosy and control patients is unclear. Because sera from controls also contained antibodies to *M. tuberculosis* which cross-reacted with determinants in the epidermis, primary sensitization to unknown antigens of bacterial or parasitic origin which are identical or similar to *M. tuberculosis* and epidermal antigens is most probable. Primary sensitization to *M. tuberculosis* with subsequent cross-reacting antibodies to epidermal determinants, and primary sensitization to *M. leprae*, with the subsequent cross-reacting antibodies to *M. tuberculosis* and epidermal determinants, is also a real possibility. Most individuals whose sera were tested came from areas where leprosy and tuberculosis are endemic. Further, primary sensitization to epidermal determinants with cross-reacting antibodies to *M. tuberculosis* cannot be excluded. In order to obtain further insight into the role of micro-organisms and the resulting cross-reacting antibodies to the epidermal determinants, it would be interesting to investigate sera from neonates for their IgM (i.e. of non-maternal origin) antibody reactivity with the extract of human epidermis in Western blot.

Further support for the idea of antigenic similarity between mycobacteria and human epidermis was obtained from our study with MAb against *M. leprae.*

Eight MAb, four of which were reactive with (epi)dermal structures in cryostat sections reacted with the extract of human epidermis in Western blot. Possibly, the same or similar antigenic determinants were detected using both techniques. Probably, MAb F 116-13 and F 116-14, reacting similarly in Western blot with *M. leprae* sonicate (12-kDa band), with human epidermis (40 kDa, 48–60 kDa [Fig. 3]) and in cryostat sections of human skin, are identical (Table 1).[2] MAb F 116-1 appeared to be different. Although showing the same reactivity with the 12-kDa protein of the *M. leprae* sonicate, it reacted differently with the epidermis in cryostat sections and with the extract of human epidermis in Western blot. Apparently a different epitope on the same 12-kDa protein, or a different 12-kDa protein, was recognized by MAb F 116-1. MAb F 116-21, directed against a 65-kDa *M. leprae* protein reacted in cryostat sections with peripheral nerve axons and with the intercellular substance in the epidermis. In Western blot, MAb F 116-21 reacted with a 40- and 47-kDa protein in the extract of human epidermis. An association between staining of the intercellular substance of the epidermis in cryostat sections and the 40-kDa protein in the extract of human epidermis in Western blot was noticed when the results obtained with MAb F 116-1, F 116-13, F 116-14 and F 116-21 were compared. Further studies with respect to the significance of this association have to be performed.

MAb F 116-4, F116-5, F 116-9 and F 116-16 reacted with the extract of human epidermis in Western blot but did not react in cryostat sections. Differences in the way in which antigen is detected in immunohistochemistry and Western blot techniques may explain this discrepancy. In the SDS–PAGE procedure 'stretching' and denaturation of protein antigens occurs which reveals antigenic determinants which may remain masked in the cryostat sections. MAb F 116-12, F 116-17, F 116-19 and F 88-1 reacted in cryostat sections, but did not react with the extract of human epidermis in Western blot. In these cases, a conformational epitope could have been lost during Western blot procedure.

The evidence suggesting mimicry between mycobacteria and epidermal antigenic determinants in this study is based on the reactivity of human antibodies and mouse MAb. In leprosy, cell-mediated and humoral immunity are simultaneously active and seem to be the determining factor in the leprosy spectrum.[11] However, CMI is emphasized because of the intracellular character of *M. leprae*. Although molecular mimicry in leprosy at the CMI level remains unproven, extrapolation of our findings on humoral immunity to CMI may be relevant. Arguments may be found in experiments with T-cell hybridomas which react with *M. tuberculosis* determinants on the 65-kDa protein, determinants which are similar to articular determinants and are involved in the pathogenesis of experimental arthritis in rat, mouse and humans.[12]

Among our hybridomas of B-cell origin, several, e.g. MAb F 116-21, were directed against the 65-kDa *M. leprae* protein. This 65-kDa protein is one of the heat shock proteins (HSPs). Recently, mouse T$\gamma\delta$ cells and human T$\gamma\delta$ cells lines reactive with the 65-kDa HSP have been isolated. Such T$\gamma\delta$ cell lines were generated from leprosy skin lesions[13]. Because evidence has emerged that stressed host cells might express HSPs on their surface[14] it may be hypothesized that in leprosy, similarity between HSPs of *M. leprae* and stressed host cells leads to expansion of T$\gamma\delta$ cells with a subsequent immune reaction to the stressed host cells, thereby removing the 'state of tolerance' of the individual.[15] Such a hypothesis would explain the severe nerve damage in tuberculoid paucibacillary leprosy patients and the frequently observed reversal reactions in borderline leprosy patients, which are accompanied by high CMI. Such nerve damage may be considered to be an autoimmune phenomenon. At the B-cell level, the characteristics of our hybridoma MAb F 116-21 support such a notion because it reacts with the 65-kDa *M. leprae* protein in Western blot, peripheral axons and epidermis in cryostat sections of the skin, and with various epidermal determinants in the extract of human epidermis using Western blot technique. The high frequency of autoantibodies in leprosy and other infectious diseases can be partly explained on the basis of antibodies against HSPs of micro-organisms which cross-react with HSPs produced by stressed host cells.[16] Further studies concerning cross-reactivity of antibodies and T-cell receptors with environmental micro-organisms and human tissues are in progress. An important aspect of the similarity in antigenic determinants between mycobacteria and (epi)dermis may be the development of skin diseases induced by environmental micro-organisms. It is well-known that a number of skin diseases are modified by infections, e.g. guttate psoriasis by streptococci[17] and erythema nodosum by chlamydiae and streptococci.[18] It is also conceivable that a number of autoimmune skin diseases such as pemphigoid, lupus erythematosus, (localized) scleroderma[19,20] and lichen planus are initiated by environmental micro-organisms. Similar considerations may also be relevant for granulomatous diseases such as sarcoidosis, granuloma annulare and Wegener's granulomatosis.

Acknowledgments

The research at the Royal Tropical Institute received financial support from the Netherlands Leprosy Relief Association. The Associazione Italiana Amici di Raoul Follereau provided financial support for the research at the Department of Dermato-Venereology in Rotterdam. The authors thank Dr B.Tank for critically reading the manuscript and correcting the English.

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British Journal of Dermatology (1992) 127, 359–364.

A quantitative immunohistochemical study of the expression of integrins by nerves in psoriatic and normal skin

S.M.ANDREW, B.D.EDWARDS,* R.J.G.CHALMERS† AND J.B.O'DRISCOLL†

Department of Pathological Sciences, Manchester University, Manchester M13 9PT, U.K.
**Department of Medicine, Manchester Royal Infirmary, Manchester M13 0WL, U.K.*
†The Skin Hospital, Salford M60 9EP, U.K.

Accepted for publication 7 May 1992

Summary Qualitative and quantitative assessment of integrin expression by dermal nerves was made by an avidin–biotin immunoperoxidase method on snap-frozen biopsies from affected psoriatic skin, and skin from normal control subjects with no history of skin disease. Nerves expressed $\alpha1$, $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$ and $\beta4$ integrin subunits, and perineural sheaths in the mid-dermis also expressed these subunits, with the exception of $\alpha2$. There were more upper dermal nerve segments expressing $\alpha1$ integrin compared with other integrins both in controls and in psoriatic skin. The greater number of nerves expressing $\alpha1$ integrin compared with other integrins may be due to anatomical or functional differences between groups of nerves. There were significantly more nerves expressing $\alpha1$, $\alpha2$, $\alpha3$, $\alpha6$ and $\beta4$ integrins in psoriatic skin compared with control skin. This generalized increase may indicate a secondary trophic effect on all nerves rather than a specific increase in one type of nerve. However, the expression of $\alpha2$ integrin may be significant in the pathogenesis of the psoriatic plaque, in that it was barely detectable in the normal site-matched biopsies, but much greater in psoriatic plaques. The study of the expression of adhesion molecules by neurones in psoriasis offers a new avenue for investigation of the role of neuronal hypertrophy in the initiation and maintenance of psoriatic plaques.

Exacerbations of psoriasis can be triggered by events such as psychological stress and localized skin trauma[1,2] and any theory of the aetiology of psoriasis should take this into account. One way in which these phenomena could be mediated is by alteration in the neurological control of epidermal turnover.[3] The origin of any alteration in neural control could be immunological, genetic, or vascular, and it is possible that it could represent the final common pathway of several different disease processes. Naukkarinen *et al.*[4] described an increase in substance P-containing nerves in lesional and non-lesional skin of psoriatics. Recently Nickoloff[5] reported that nerve endings similar to those containing substance P can be labelled with monoclonal antibody to the $\beta4$ subunit of the integrin family of adhesion molecules.

The integrins are a recently characterized group of cell-surface glycoproteins expressed in the form of non-covalently associated α/β heterodimers, which serve both a cell–cell and cell–matrix receptor function. There is increasing experimental evidence that integrins influence intercellular adhesive events including those occurring during neural development,[6] in the spatial organization and terminal differentiation of the epidermis,[7] in leucocyte adherence to endothelium at sites of inflammation, and in antigen presentation.[8] In addition to the biological interest in the expression of adhesion molecules by tissues, the expression of different integrins by neural structures is of interest because they are bound to the cell membrane and thus show good spatial localization around the cell periphery, and section staining by immunocytochemistry gives a distinct, crisp cellular outline. This enables reliable measurement of nerve length in contrast with neurotransmitter staining, because neurotransmitters are unevenly distributed through the cell and nerves stained for them can be difficult to follow. We report our findings of the expression of a wide range of integrins on neural structures in the skin.

Methods

Punch biopsy specimens (4 mm) were obtained from the gluteal region of normal volunteers ($n=4$), from the

Table 1. Primary antibodies used

MAb	Working dilution	Integrin subunit	Raised in	Origin
A-1A5	1/1000	$\beta1$	Mouse	Hemler[10]
TS2/7	1/500	$\alpha1$	Mouse	Hemler[11]
12F1	1/500	$\alpha2$	Mouse	Pischel[12]
J143	1/500	$\alpha3$	Mouse	Fradet[13]
BG10	1/500	$\alpha4$	Mouse	Hemler[14]
BIIG2	1/10	$\alpha5$	Rat	Brown and Juliano[15]
GoH3	1/30	$\alpha6$	Rat	Sonnenberg[16]
TS/18	1/1000	$\beta2$	Mouse	Springer[17]
MAb15	1/1000	$\beta3$	Mouse	Ginsberg*
439-9B	1/50	$\beta4$	Rat	Falconi[18]

* Gift from M.H.Ginsberg, Scripps Clinic and Research Foundation, La Jolla, U.S.A.

chest skin of patients undergoing thoracic surgery ($n=4$) and from lesions in the gluteal region of patients with untreated chronic plaque psoriasis ($n=12$). Informed consent was given by each patient. All subjects were in the age range 30–50 years. The specimens were covered in OCT cryoprotectant (Tissue Tek) and snap-frozen in liquid nitrogen. Cryostat sections (5 μm) were fixed in acetone for 5 min and endogenous peroxidase activity was blocked according to Andrew and Jasani.[9] Monoclonal antibodies to various integrin subunits (Table 1) were applied to the sections at optimal concentrations. The secondary antibodies were biotinylated anti-mouse or rat antibody (Sigma) as appropriate, and the labelling was visualized using an avidin–biotin kit (ABC Kit, Dako) with diaminobenzidine as the substrate.

Semi-automated quantification was performed on a Leitz Laborlux microscope attached to a Hitachi KP-123 camera, which input into a Tandon PC with a 286 processor running a Freelance (Sight Systems, Foster Findlay Assoc.) image analysis programme. Nerve lengths lying within an area defined on the image analyser were measured, and results expressed as the sum of nerve lengths in millimetres per unit area (mm^2). The measurements were all made in the upper dermis, which for the purpose of this study was defined as that part of the dermis lying within one high-power field of the dermo-epidermal junction (× 25 objective lens, with the magnification in the camera giving a final magnification of × 400). Statistical comparison was made using Student's two-tailed *t*-test.

Results

Integrin staining allowed clear visualization of nerves in the skin. Nerves were seen in normal and psoriatic skin expressing $\beta1$, $\alpha1$ (Fig. 1), $\alpha2$, $\alpha3$ (Fig. 2), $\alpha6$ and $\beta4$ integrins. With the exception of $\alpha2$ the connective tissue sheaths of nerve bundles in the mid- and lower dermis expressed the same integrin subunits as the nerves (Table 2). Beta-2, $\beta3$, $\alpha4$ and $\alpha5$ integrins were not expressed by any nerves. Dendritic cells of the epidermis and papillary dermis expressed $\alpha4$ and $\beta2$ antigens.

A quantitative assessment was made of the staining of

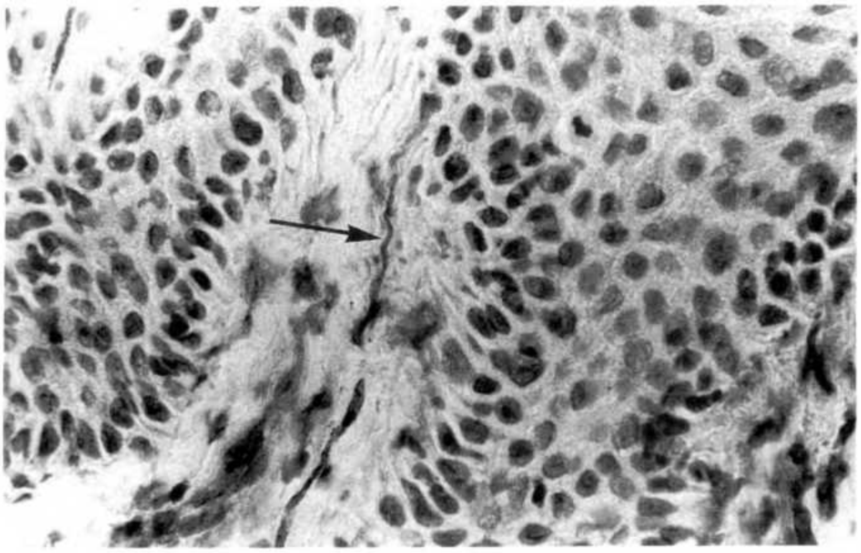

Figure 1. Expression of $\alpha1$ integrin by a small nerve (arrow) in the upper dermis of a psoriatic plaque. This integrin is not expressed by keratinocytes but is expressed by dermal cells including lymphocytes, which, although present in the sections have a different structure from the nerves and therefore did not interfere with quantification.

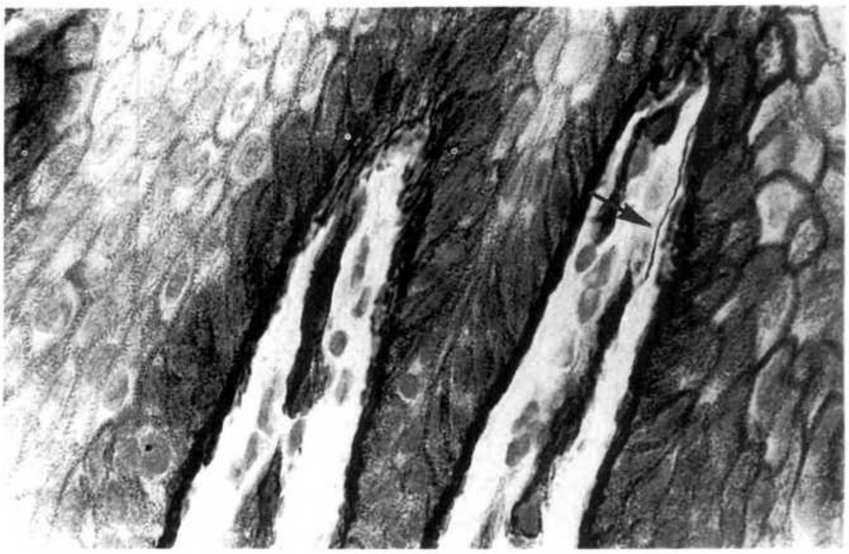

Figure 2. Expression of α3 integrin by a very fine, but relatively long nerve (arrow) in the upper dermis of a psoriatic plaque. This integrin is also heavily expressed by keratinocytes and endothelial cells, which are easily distinguished from nerves by their different structure.

Table 2. Integrin expression by cutaneous nerves and perineurium

	Integrin subunit							
Neural structure	β1	α1	α2	α3	α4	α5	α6	β4
Deep dermal neural bundles	++	++	+	+	−	−	+	+
Perineurium of neural bundles	++	++	−	++	−	−	++	++
Small (2–5 μm) upper dermal fibres	++	++	+	+	−	−	+	+
Very small (< 2 μm) fibres	++	++	+	+	−	−	+	+

++, maximum staining of nerves; +, fewer number of fibres stained; −, no fibres stained.

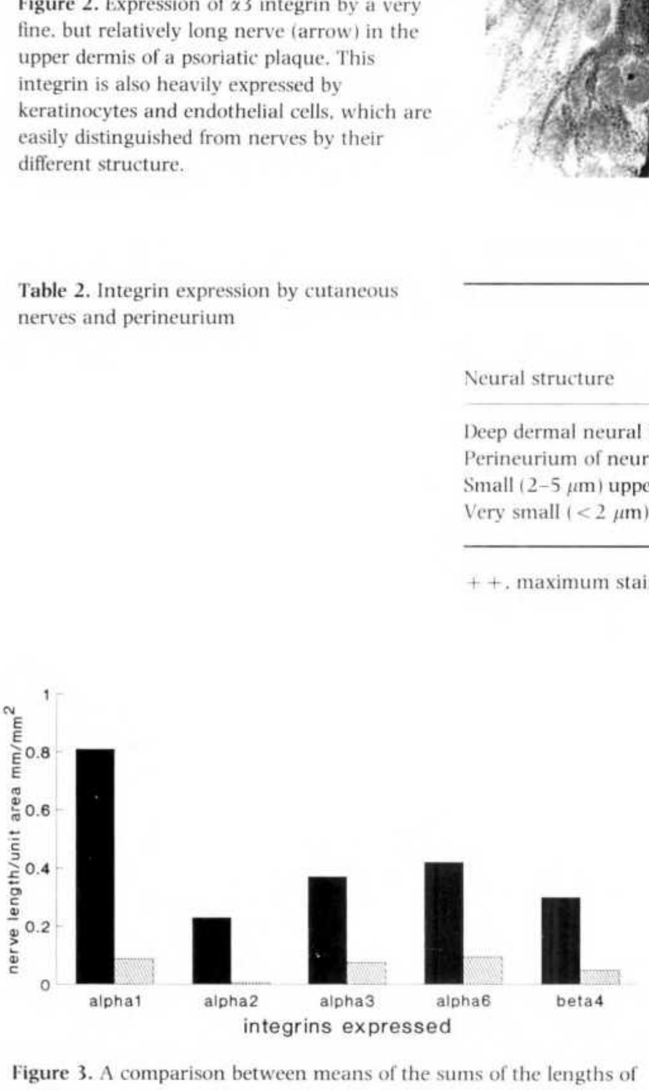

Figure 3. A comparison between means of the sums of the lengths of small (< 5 μm diameter) nerves present in the upper dermis (mm of nerves per mm^2 of papillary dermis) of biopsies of psoriatic plaques ($n = 12$) and biopsies from control subjects ($n = 4$). The mean sum of nerve lengths is higher in psoriatic plaques than controls in the sections stained for α1, α2, α3, α6 and β4 integrins (α1, α2, α3, α6, $P < 0{\cdot}01$; β4, $P = 0{\cdot}02$: two-tailed *t*-test). The mean of the sum of nerve lengths per mm^2 expressing α1 integrin is higher than that of nerve lengths expressing other integrins ($P < 0{\cdot}02$ in all cases: two-tailed *t*-test). ■, cases; ▨, controls.

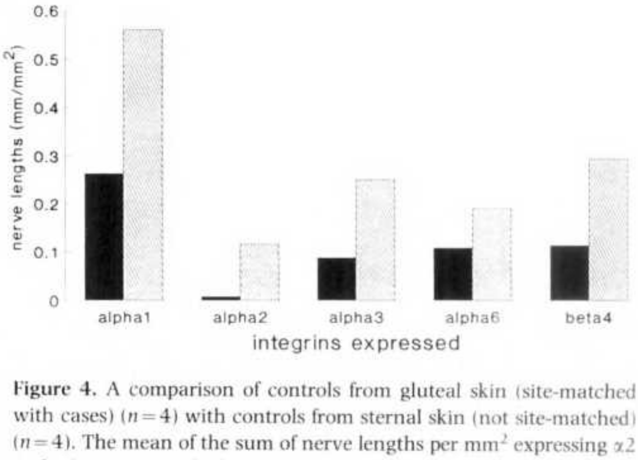

Figure 4. A comparison of controls from gluteal skin (site-matched with cases) ($n = 4$) with controls from sternal skin (not site-matched) ($n = 4$). The mean of the sum of nerve lengths per mm^2 expressing α2 and α3 integrin is higher in the sternal skin than the gluteal skin ($P < 0{\cdot}05$: two-tailed *t*-test). ■, gluteal; ▨, sternal.

small nerves (< 5 μm diam.) in the upper dermis by all the integrins expressed, except $\beta 1$. Heavy staining of inflammatory cells by $\beta 1$ obscured some of the nerves, making quantification difficult, and it was not attempted. Counts of nerve lengths obtained from observations on the control gluteal skin ($n=4$) compared with nerve lengths measured in psoriatic plaques ($n=12$) showed that there were more of these nerves in the psoriatic skin than in the controls (Fig. 3). In all the biopsies there were more upper dermal nerves expressing $\alpha 1$ integrin than nerves expressing any other integrin (Fig. 3).

A difference was found between the number of nerves in the gluteal skin (site-matched with the biopsies) controls ($n=4$) compared with those from the chest ($n=4$), there being more nerve lengths expressing $\alpha 1$, $\alpha 2$, and $\alpha 3$ in the skin from the chest ($P<0{\cdot}5$; Fig. 4).

Discussion

Psoriasis is a disease which affects the entire skin, not merely areas which are clinically abnormal. The involvement of an area of skin by active disease can be triggered by trauma and stress. Psoriatic plaques are often strikingly symmetrical. Cases of traumatic denervation of an area of skin have been described in which psoriasis cleared from the denervated skin.[2] This suggests that the distribution of lesions may be influenced by neural input.

The nerve supply of the skin is from deep and superficial plexuses. Large nerve bundles occur mostly in the lower and mid-dermis, and small nerves, which may be myelinated or unmyelinated, are seen in the papillary dermis and around appendages. The only anatomical structures in the upper dermis that could possibly be confused with small nerves and their processes are dendritic cells. The structures described in this study are thought to be of true neural origin because of their morphology, and because they do not express the two integrins typical of dendritic cells ($\alpha 4$ and $\beta 2$). The measurements taken in this study were of segments of neurones present in 5-μm sections and so are not measurements of whole cells, but of several parts of one or more cells. We have demonstrated that the different integrins stain different numbers of neurone segments in the dermis of control and psoriatic skin.

Peripheral nerves contain various collagen and laminin.[20,21] Laminin and types IV and V collagens in particular are present in the endoneurial basement membrane and are therefore intimately associated with the neuronal cell membrane. The endoneurial basement membrane is synthesized by Schwann cells and is absent from bare neural endings.[22] Alpha-$1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins are known to function as extracellular matrix receptors for laminin and/or collagen on neuronal cell lines in culture.[23-25] In addition, $\alpha 1/\beta 1$ has been shown to be a receptor for type IV collagen[26] and a mediator of axonal growth of sympathetic neurones on a type IV collagen substrate.[27]

We have found that the most striking difference in the expression of integrins is that $\alpha 1$ is expressed by more nerves than are the other integrins. One explanation for the excess of $\alpha 1$-positive neurones could be that this receptor is expressed by the entire length of the neurone, whereas the others are confined to the part of the neurone enclosed by the basement membrane of the Schwann cell. However, the possibility of the expression of $\alpha 2$ and $\alpha 3$ being confined to one subset of functionally different nerves cannot be excluded.

There is evidence for accelerated turnover of nerves in affected and unaffected psoriatic skin,[2,4] and psoriatic plaques are more densely innervated than unaffected psoriatic or control skin.[27] Our study has confirmed that nerves in the upper dermis are increased in psoriatic skin compared with control skin taken from the same area of the body of patients with no history of skin disease. The higher numbers of nerves seen in the psoriatic biopsies compared with controls could be due either to neuronal reduplication, which is unlikely, or to sprouting of new processes by pre-existing neurones.[28] In the present study we demonstrated that all subsets of nerves were increased in the psoriatic biopsies compared with site-matched controls. This may indicate that there is a non-specific trophic effect upon all the nerves. However, the expression of $\alpha 2$ integrin is particularly interesting in that it is barely detectable in normal gluteal skin but much more prominent in psoriatic skin. This may represent a subset of nerves which is relatively rare (compared with other nerve subsets) in normal skin, but increased in psoriatic skin, and which may be important in the pathogenesis of psoriatic plaques.

The identity of the ligand for $\beta 4$ integrin is controversial. It is thought that in the epidermis $\alpha 6\beta 4$ localizes to hemidesmosomes and that $\beta 4$, which has a particularly long cytoplasmic domain, is important in the interaction of the transmembrane part of the hemidesmosome with the anchoring filaments.[29] However there are no hemidesmosomes in nerve, and the function of $\beta 4$ integrin in nerves is unknown.

Nickoloff[5] proposed that $\beta 4$-positive nerves have a similar structure to substance P-containing nerves. Neuropeptides appear to play a part in the regulation of cutaneous inflammation, pain and pruritus, and substance P acts as a neurotransmitter in the mediation of neurogenic inflammation.[30,31] Elevated levels of both substance P and VIP have been demonstrated by radioimmunoassay of psoriatic lesional skin compared with non-lesional and control skin, but difficulty has been experienced in confirming this by immunohistochemistry on matched samples.[32] However, increased substance P production is not specifically associated with psoriasis, as it has been found in other inflammatory skin diseases.[33] It would be interesting to examine the localization of neuropeptides in nerves labelled with integrins to assess whether the different integrins are expressed in nerves with different functions.

The importance of exercising care in the selection of controls for dermatological studies is demonstrated by the presence of an excess of nerves in chest skin compared with gluteal skin. The excess is due to the naturally denser sensory innervation of chest skin compared with gluteal skin, and possibly to the different requirement of sternal skin for effector innervation.

In summary, we have found that integrins are expressed on nerves in normal and psoriatic skin and that they may serve as markers of neural structures. The excess of integrin-positive nerves is seen with all subsets of integrin expressed by nerves, probably indicating a generalized trophic effect on the nerves within plaques. However, the expression of $\alpha 2$ integrin appeared particularly prominent in that it was barely detectable in the normal site-matched biopsies. It may therefore be a marker for a pathogenetically significant subset of nerves within psoriatic plaques. Further studies are needed to localize precisely the site of the integrin molecules on different nerves at the ultrastructural level, and studies with combined integrin and neurotransmitter antibodies may help to unravel the complex interactions of dermal neural structures and the control of epidermal proliferation.

Acknowledgments

We are grateful to the many investigators who generously provided us with the antibodies needed for this study. The antibodies were obtained from M.E. Hemler, Dana-Farber Cancer Institute, Boston, U.S.A.; V.L.Woods, UCA San Diego, U.S.A.; T. Albino, Memorial Sloane Kettering Institute, New York, U.S.A.; C.Damsky, UCA San Francisco, U.S.A.; A.Sonnenberg, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands; T.A.Springer, Center for Blood Research, Boston, U.S.A.; M.H.Ginsberg, Scripps Clinic and Research Foundation, La Jolla, U.S.A; S.J.Kennel, Oak Ridge National Laboratory, Oak Ridge, U.S.A..

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British Journal of Dermatology (1992) 127, 365–371.

Peripheral anchorage of dermal equivalents

C.A.LÓPEZ VALLE,*† F.A.AUGER,*‡ P.ROMPRÉ,*§ VÉRONIQUE BOUVARD* AND LUCIE GERMAIN*‡

*Skin Culture Laboratory, Saint-Sacrement Hospital, Quebec, Quebec, Canada, G1S 4L8
†Department of Microbiology, Laval University, Sainte-Foy, Quebec, Canada, G1K 7P4
‡Department of Surgery, Laval University, Sainte-Foy, Quebec, Canada, G1K 7P4
§Department of Chemical Engineering, Laval University, Sainte-Foy, Quebec, Canada, G1K 7P4

Accepted for publication 17 April 1992

Summary

Human fibroblasts can induce collagen gel contraction with different kinetics depending on the number of cells and on the collagen concentration within this lattice, which has been considered as a dermal equivalent. Skin equivalent is a combined culture of dermo-epidermal layers which may be of therapeutic value in the treatment of burn patients. However, the current production of the dermal equivalent component gives results that present many drawbacks for their eventual clinical use as a first step in obtaining a skin equivalent. These include: (i) final surfaces which are very small; less than 20% of the initial size (ii) excessive thickness which may hamper successful graft take (iii) fibroblasts that do not have an arrangement comparable with normal dermal tissue.

We propose, as a solution to these problems, the utilization of a 5-mm-wide fibre-glass filter ring peripherally attached to the surface of the Petri dishes to prevent inordinate contraction while the fibroblasts reorganize the collagen gel. Using this technique the initial surface was preserved and the dermal equivalent contracted only in thickness. Histological analysis of these anchored equivalents confirmed an alignment of fibroblasts and collagen fibres resembling normal dermal tissue. We consider this method useful in the development of dermo-epidermal sheets for clinical purposes.

The various techniques developed for the culture of epidermal sheets have allowed an important breakthrough in the treatment of large burn wounds.[1,2] However these grafts, although they provide adequate and permanent coverage, are incomplete, because they lack the dermal component of skin. The absence of dermis may be an important drawback because the dermal layer adds mechanical properties such as constant tension, shock resistance, elasticity and extensibility[3,4] to the protective barrier quality of the epidermis.

Histological analysis of transplanted epidermal grafts has shown that 'neodermis' formation under these grafts is not present during the first year after grafting.[5] This delay of at least 1 year in tissue reorganization may be obviated by grafting dermo-epidermal sheets, thus eventually reducing the scar tissue formation stage.

The utilization of collagen gel as a matrix for growing cells was first described in 1956.[6] Since then, modifications of this culture technique have been suggested to allow cell growth in a 3-dimensional environment, which could be experimentally altered to resemble natural and pathological conditions.[6] In 1979 a method was reported of using collagen to recreate a dermis-like structure *in vitro*.[7] Subsequently, the culture of skin equivalents (SE) has been extensively described.[8–11]

The SE consists of two components: the dermal equivalent (DE) which is prepared first, and the epidermal equivalent which is recreated by applying a suspension of keratinocytes on to the DE. The critical step in the SE technique is the DE formation, which is obtained by mixing fibroblasts with a collagen solution. This solution will gel after a few minutes, according to the physicochemical conditions.[12,13] The incorporated fibroblasts actively reorganize the collagen fibres and induce a dramatic contraction.[7,10] The final surface of the DE is inversely proportional to the number of cells, and directly proportional to the collagen concentration.[7] Thus, some authors have proposed that a judicious ratio of cell to collagen concentration in the DE may be selected in order to limit the amount of contraction. However, our own experience has proved otherwise, i.e. that DE contraction is quite severe. Furthermore, the addition of keratinocytes to obtain a SE will always further reduce the final surface in a concentration-

Correspondence: Dr F.Auger, Laboratoire de recherche des grands brûlés, Hôpital du Saint-Sacrement, 1050 chemin Sainte-Foy, Québec, Canada, G1S 4L8.

dependent manner.[14–16] All keratinocyte concentrations giving histologically acceptable SE lead to an important additional contraction (final surface $\leq 20\%$ of the initial surface).[14] The mechanisms by which the keratinocytes induce such a contraction are not completely understood, although these cells can interact with native collagen fibrils and reorganize the network in the absence of fibroblasts[17] independently from serum and fibronectin.[18]

Thus the production of a clinically useful bilayered SE presents a formidable challenge. Therefore, we have developed a new technique for obtaining DE with a minimal level of contraction. The addition to the culture dishes of a peripheral ring made of filter material enabled a firm anchorage of these DE. The contraction phenomenon induced by the fibroblasts contained in the collagen gel reduced the thickness but not the DE surface. Furthermore, histological analysis of these anchored dermal equivalents (ADE) demonstrated a cellular-fibrillar arrangement much closer to normal skin histology[19] than any previous technique.

Methods

Medium preparation

A combination of Dulbecco–Vogt modification of Eagle's medium (DME) with Ham's F12 in a 3:1 proportion (Flow Labs, Mississauga, Ont., Canada) was used as the base medium. To each litre of medium 24·3 mg of adenine (Sigma Chemical, St Louis, MO, U.S.A.) was added. The medium was sterilized through 0·22-μm filters, Millipak 40 (Millipore, Bedford, MA, U.S.A.) and brought to pH 7·35–7·45. Before use this medium was supplemented with 5 μg/ml bovine crystallized insulin (Sigma), 5 μg/ml human transferrin (Sigma), 0·4 μg/ml hydrocortisone (Calbiochem, CA, U.S.A.), 10^{-10} M cholera toxin (Schwarz/Mann, Cleveland, OH, U.S.A.), 10% fetal calf serum (Sigma), 10 ng/ml human EGF (Chiron Corp., Emeryville, CA, U.S.A.), 100 IU/ml penicillin G (Sigma) and 100 μg/ml streptomycin sulphate (Sigma). This complete medium formulation is based on conditions developed for serial cultivation of keratinocytes.[20–23]

Fibroblast cultures

Fibroblast cultures were established from skin obtained at the time of reductive breast surgery. The cells were seeded at 1×10^6 per flask and propagated in the previously described supplemented medium. These cells were inoculated in 75 cm^2 Falcon tissue culture flasks (Becton Dickinson, Mississauga, Ont., Canada) and were kept at 37°C in an 8% CO_2 atmosphere.

Cells were resuspended just before confluence with a solution of 0·01% EDTA and 0·05% trypsin in PBS. A sample was taken to determine the number of cells obtained. The suspension was centrifuged at 300 *g* for 10 min and a final count performed before bringing the suspension, in DME-HAM, to the desired concentration.

Collagen solution

Acid-soluble type I collagen, from calf skin (Sigma type III) was dissolved overnight at 4°C in 1:1000 acetic acid solution to a final concentration of 2·11 mg/ml or 4·22 mg/ml.

Preparation of anchoring Petri dishes

Two 5 mm-wide rings were cut from a glass microfibre filter 934-AH (Whatman, Maidstone, U.K.). These were glued together and attached to the bottom of 81-cm^2 square bacteriological Petri dishes using epoxy cement. The dishes were then resterilized in ethylene oxide.

Control floating dermal equivalents were prepared in standard bacteriological Petri dishes.[7]

Preparation of dermal equivalents

Fibroblast-populated collagen gels were prepared in 50-ml centrifugation tubes (Sarstedt, Ville St-Laurent, QC, Canada) with various collagen and cell ratios. These mixtures were poured into 81-cm^2 LabTek bacteriological dishes, with or without the fibre-glass filter ring. Each dish contained 5·6 ml of ×2·7 concentrated DME medium with penicillin and streptomycin, 3·7 ml of fetal calf serum, 9·5 ml of collagen solution, 0·15 ml of 0·1 N NaOH, and 1 ml of fibroblasts suspended in ×1 DME-HAM. Dishes were incubated at 37°C in an 8% CO_2/92% air atmosphere. Gels were completely set after 10 min. Complete medium was used for media changes. Media changes were initiated on the third day after DE preparation and subsequently performed each third day.

Evaluation of dermal equivalent dimensions

To calculate dermal equivalent dimensions, all Petri dishes were placed on a glass surface 7 cm above an X-ray viewbox. A piece of black cardboard was used on the viewbox's screen to obtain indirect transillumination. Square floating dermal equivalents (FDE) showed slight differences in side length. Photographs of each equivalent were taken daily. The standardized pictures produced were read with planimetric scales to obtain the daily surface of each equivalent. Peripherally anchored dermal equivalent surfaces did not change. All results are given as a percentage of the initial surface.

Biopsies of dermal equivalents

At least two punch biopsies of 1 cm diam. were taken from different sites on each dermal equivalent. Biopsies were fixed in Bouin's solution and embedded in paraffin in a standard manner, followed by staining with haematoxylin and eosin for cell analysis. Masson's trichrome was employed for collagen pattern visualization.

Thickness measurements

The thickness measurements of all dermal equivalents were performed by optical microscopy of histological slides. A Leitz Wetzlar (Germany) eyepiece with cross reticulation (10 mm/100 divisions) was employed. All dermal equivalents showed thickness variations. Therefore, we performed five measurements on each equivalent and the mean result with the SD is included in Table 1.

Results

Floating dermal equivalent formation

Under phase-contrast microscopy, round, trypsinized fibroblasts began to show cell processes a few minutes after gel setting. Two hours later the majority of cells became stellate. After a short time-lag, contraction started more or less rapidly, depending on cell number and collagen concentration.

The initial contraction speed varied in relation to cell number. Gels with 18,500 fibroblasts/cm^2 showed a reduction to 4% of the original surface area at 24 h while those containing 245 cells/cm^2 showed a mean reduction to 85% of the original surface area during the same period. By the fourth day most of the contraction process had occurred, and the original surface area was reduced to 2 and 45%, respectively, in the previously described conditions. No further contraction was observed after the sixteenth day in any experimental condition, and the mean final surfaces were less than 1 and 35% for the above mentioned cell numbers. The results showing the relationship between floating DE surfaces and various cell concentrations are summarized in Figure 1.

As was shown previously,[7] the optical properties of collagen gels change during contraction. They become progressively more opaque and eventually whitish-yellow. Furthermore, the consistency of dermal equivalents varied depending on cell numbers and collagen concentration.

Increase of the collagen concentration was accompanied by a reduction in the contraction rate and by an augmentation of the final surface. Figure 2 shows an example of the influence of collagen concentration.

Peripherally anchored dermal equivalent formation

Changes in cell morphology were similar to those observed in floating dermal equivalent formation as observed by phase-contrast microscopy. After gelation, cells were evenly sprinkled throughout the DE. Refringent fibres appeared within 24 h connecting cells in a circular pattern. Our observations suggest that daughter

Table 1. Thickness of related FDE and ADE in μm*

	Collagen concentration (mg/ml)	Fibroblasts/cm^2			
		18,000	1235	617	555
FDE	2.11	1499±168	1345±91	1169±109	782±117
ADE	2.11	316±51	307±58	326±65	286±36
FDE	4.22	1120±155	ND	ND	ND
ADE	4.22	358±92	ND	ND	ND

*Expressed as mean±SD

ND=not determined.

FDE were thicker than ADE in related experimental conditions. Random analysis showed that the anchoring method induced formation of thinner dermal equivalents than the floating method. Initial volume was 20 ml and initial surface was 81 cm^2 for both FDE and ADE.

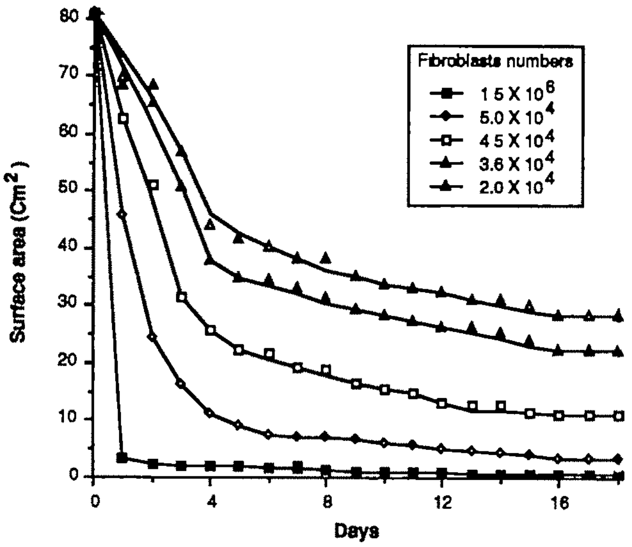

Figure 1. Influence of fibroblasts numbers on final surface. The initial contraction speed varied in relation to cell numbers. By the fourth day most of the contraction process had occurred. No further contraction was observed after the sixteenth day in any experimental condition. Collagen concentration was 2·11 mg/ml.

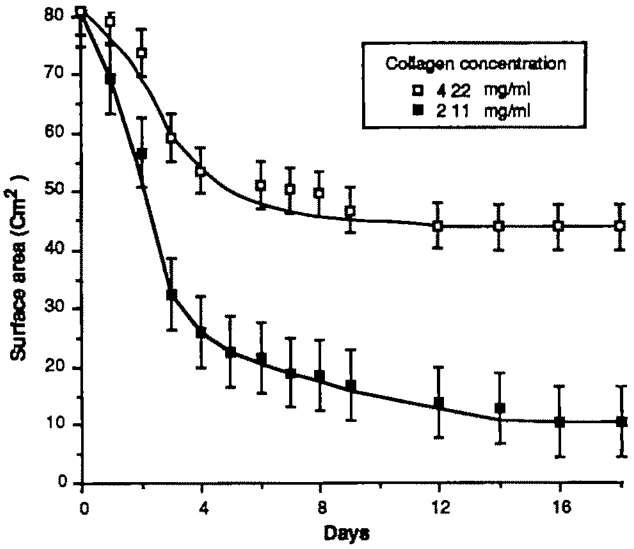

Figure 2. Influence of collagen concentration on final surface Increase of the collagen concentration was accompanied by a reduction in the contraction rate and by an augmentation of the final surface. Total fibroblast number was constant, 4·5 × 10⁴ (initial concentration of 555 cells/cm²) Bars represent SEM from three replicates.

cells formed bundles in this original arrangement, and this increasing number of fibroblasts began outward migration in all directions (Fig. 3). At the final stage, cells were arranged in a complex three-dimensional circular array, with most cells parallel to the surface of the Petri dish. No surface reduction was noted when the fibre-glass filter ring was used.

Anchored dermal equivalents with a collagen concentration of 2·11 mg/ml and containing 18,500 fibroblasts/cm² had a tendency to tear in their central portion. This phenomenon disappeared when the collagen concentration was raised to 4·22 mg/ml for the same cell number.

A progressive opacity and solidification of the gel were also observed macroscopically, according to culture time.

Floating dermal equivalents (FDE)

Histology of floating dermal equivalents synthesized with different cell numbers and collagen concentrations presented some common characteristics.

1. A fibroblast multilayer enveloped both surfaces of floating equivalents. This phenomenon did not occur when the cell number was lower than 444 fibroblasts/cm².
2. Fibroblasts within the FDE were orientated perpendicular to tissue length, whereas those present at the FDE surface were parallel to tissue length (Fig. 4b).
3. Collagen fibres were orientated perpendicular to the surface (Fig. 4b). This histological feature was not observed when the fibroblast numbers were ≤ 617 cells/cm².
4. The surface of floating dermal equivalents containing more than 1235 cells/cm² showed many folds. These folds were filled with colonies of fibroblasts (Fig. 4a).

Anchored dermal equivalents (ADE)

Histology of peripherally anchored dermal equivalents differed from the floating equivalents.

1. All experimental conditions showed an absence of a fibroblast multilayer envelope around the matrix.
2. Fibroblasts were orientated longitudinally to tissue length (Fig. 5b).
3. Collagen fibre orientation was mostly longitudinal in alignment (Fig. 5b).
4. No folds were observed.

A microscopic study of the fibre-glass filter ring in the ADE showed penetration of the collagen into the interstices of this material. The anchoring phenomenon seemed to be related to its entrapment in the porous

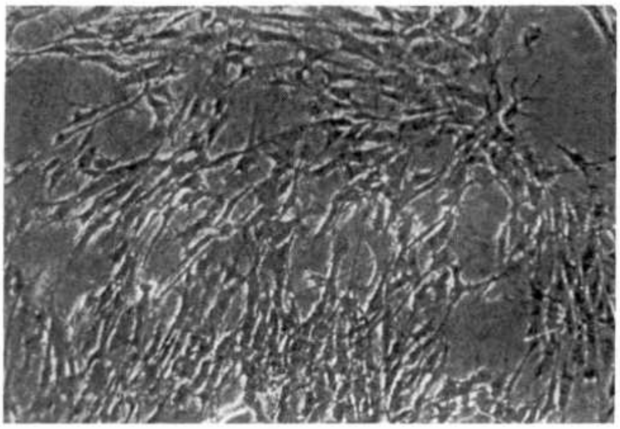

Figure 3. Living peripherally anchored dermal equivalent containing $1{\cdot}5 \times 10^6$ fibroblasts at 48 h in culture (18,000 cells/cm^2). Cells arranged in a circular pattern. Daughter cells began migration outward in all directions. View from below using phase contrast microscopy (× 135).

structure, rather than to any cellular binding, as very few cells were seen within the fibre-glass ring (Fig. 5c).

Stability of peripherally anchored equivalents

A set of experiments was carried out to evaluate the stability of these ADE after their detachment from the filter ring. A contraction of approximately 10% occurred within 3 days after detachment in dermal equivalents with 18,500 fibroblasts/cm^2 (Fig. 6).

Thickness measurements

Fifty-two floating dermal equivalents were compared with 44 anchored dermal equivalents. Random analysis showed a mean thickness of 673 μm for FDE, with a range of 151–1560 μm. Mean thickness for ADE was 348 μm with a range of 100–496 μm. Comparison of some related experimental conditions are presented in Table 1.

Discussion

This study was initiated to evaluate whether a peripheral anchoring technique might solve the inherent deficiencies in the usual methods of recreating a DE *in vitro*. The results obtained with the FDE technique illustrate these pitfalls.

The FDE technique has invariably produced final surfaces that were so small that they precluded their use as clinically significant grafts for burn therapy. Lowering fibroblast numbers in these collagen lattices lessened this shrinkage phenomenon.[7,14,24] However, the resultant DE was then so poorly organized that it was very fragile. Furthermore, the histological resemblance to normal dermis was lost.

One proposed solution to circumvent the shrinkage problem is the use of much larger tissue culture flasks. There are, however, practical limits to this approach, and the initial surface would have to be extremely large. For example, to obtain a 5 × 8 cm graft, considering a conservative 95% shrinkage level, an initial culture dish of 100 × 160 cm would be necessary. These conditions are impractical and preclude the possibility of any scaling up of operations. In addition, if a solution of keratinocytes is then 'plated' on these DE to obtain SE, a further reduction of the final surface will ensue which will greatly compound the problem.[14,15]

The resultant FDE are relatively thick, and this may have significant effects on the process of angiogenesis which normally occurs after transplantation. As showed by Yannas, using cell-free polymeric membranes, this ingrowth process could take between 10 and 46 days after application, depending mainly on thickness, chemical composition and type of polymerization.[25–27] Interference with vascularization lessens the viability of the

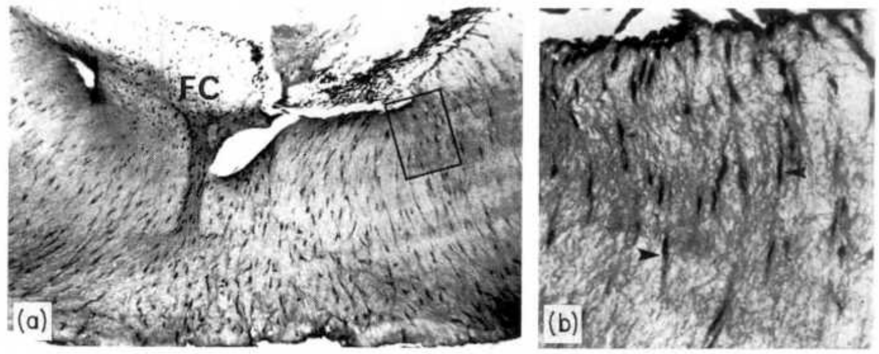

Figure 4. (a) Histological appearance of full thickness FDE showing an envelope of fibroblasts mainly on the surface, and one fold filled by a colony of fibroblasts (FC). Initial collagen concentration was 2·11 mg/ml and fibroblast number was 18,500/cm^2 (Haematoxylin and eosin. Taken at × 20 and enlarged photographically to × 40.)
(b) Enlargement of the indicated portion showing fibroblasts (arrowheads) and collagen fibres orientated perpendicular to tissue length. (Taken at × 80 and enlarged photographically to × 160.)

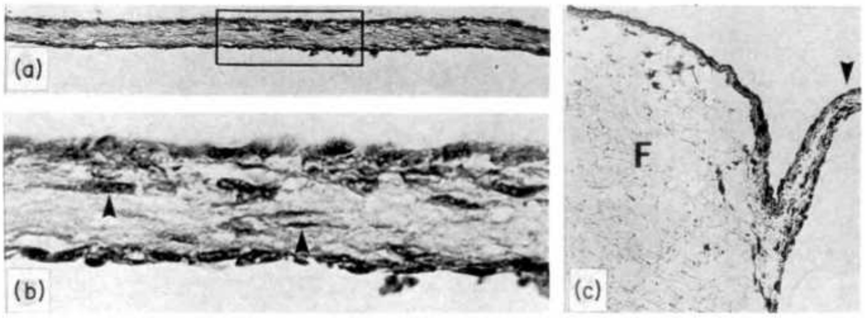

Figure 5. (a) Histological appearance of full thickness ADE. Comparison with Figure 4(a) shows that the thickness of the ADE is less for the same conditions (initial cell and collagen concentrations). Initial collagen concentration was 2·11 mg/ml and fibroblast number was 18,500/cm^2. (Haematoxylin and eosin. Taken at × 20 and enlarged photographically to × 40.) (b) Enlargement of the indicated portion of Figure 5(a) showing fibroblasts (arrowheads) and collagen fibres orientated longitudinally in relation to tissue length. (Taken at × 40 and enlarged photographically to × 160.) (c) Histology of the fibre-glass ring (F) interface with the ADE (arrowhead). Haematoxylin and eosin staining. (Taken at × 20 and enlarged photographically to × 40.)

overlying epidermis and results in a lower take level. However, this was not noted in previously described experiments using SE,[9,10] but it is possible that the small size of these grafts permitted their lateral nourishment.

Lastly, our histological analysis of these FDE has shown that their cellular and fibrillar arrangement, mostly perpendicular to the surface, is not comparable with normal human dermis.[19] It seems logical to strive for DE with histological features as close as possible to the normal dermis.

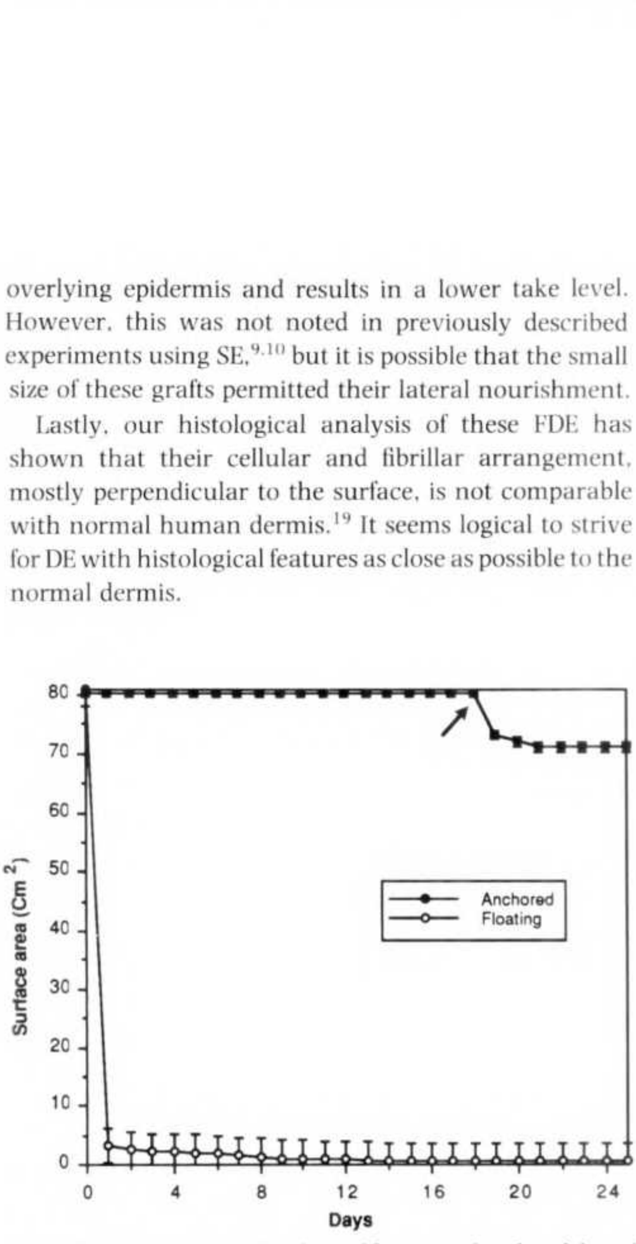

Figure 6. Comparative final surfaces of floating and anchored dermal equivalents. Floating dermal equivalent surface: 0·74%, anchored dermal equivalent surface after freeing: 87·8%. Collagen concentration 2·11 mg/ml, fibroblasts numbers: 18,500/cm^2. Arrow indicates the freeing day. Bars represent SEM from three replicates.

Our results show that the anchoring of the dermal equivalent is a practical solution to the drawbacks of the FDE. In their studies of tissue morphogenesis, Harris *et al.* described a related technique for stabilizing collagen gels devoid of incorporated cells. Fibroblasts were subsequently added to the surface of these collagen gels. The objective of these studies was the analysis of the generation of fibrillar patterns created by various mechanical instabilities,[28–30] whereas we wished to obtain large and stable DE. We have shown that the peripheral anchoring technique can achieve such results without complicated manipulations of the culture conditions. Anchorage transformed the contraction process into a unidimensional rather than a three-dimensional phenomenon. The only axis of shrinkage involved the thickness of the ADE because it was the only axis along which the fibroblasts could act freely. As previously discussed, we feel that a thinner DE may facilitate graft take and the survival of the keratinocytes contained in the SE. This cultured SE has to be nourished by osmosis during the first few days after grafting, as are standard grafts.[31]

The histological features of the ADE are also much closer to normal dermis.[19] The longitudinal arrangement of cells and fibrils in ADE is quite remarkable.

Our observations suggest that the attachment of the ADE to the filter ring is a fibrillar entrapment phenomenon rather than a cellular binding mechanism. Thus, it is possible that any porous material will permit efficacious anchoring of dermal equivalents. The choice of an optimal material would be dictated by its biocompatibility, by its ease of use and its resistance to sterilization, allowing repeated utilization.

We believe that the ADE model presented in this report is a first step in the production of new skin substitutes for treatment of burn patients by tissue culture methods. The addition of other molecules in the initial mixture could be helpful in obtaining a more complex and structured dermal equivalent. Such an anchoring technique could be readily amenable to large-scale production of skin equivalent for wound coverage of burns.

Acknowledgments

This work was supported by Grant No. MA-10223 from the Medical Research Council of Canada, Award B-2 committee 01A from the Fonds FCAR (Québec) to C.A.López Valle and Award PGS 1 from Natural Sciences and Engineering Research Council of Canada to P.Rompré, and Fellowships from the Fonds de la Recherche en Santé du Québec (FRSQ) to F.A.Auger and L.Germain.

We thank Dr George Nascimento from Chiron Corporation for graciously supplying EGF, Céline D.Fugère for assistance with histology and Jo-Anne Massé for help with technical work.

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British Journal of Dermatology (1992) 127, 372–378.

Isolation and characterization of a lower molecular weight protein doublet of horny cell outer leaflet: a possible novel epidermal differentiation marker

J.K.PARK,* T.YOSHIIKE, H.YAGUCHI AND H.OGAWA
Department of Dermatology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyoku, Tokyo 113, Japan

Accepted for publication 21 April 1992

Summary The water-soluble fraction of stratum corneum contains numerous unidentifiable proteins and polypeptides when examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Intensely stained polypeptide bands of low molecular weights (LMW) [8 and 10 kDa] are also present, and the abundance and invariable presence of this LMW protein doublet in the soluble proteins of stratum corneum prompted us to isolate and characterize them. Extracts of stratum corneum, using 8 M urea, 0·05 M Tris, pH 9·0, 0·025 M 2-mercaptoethanol, were dialysed against water. Water-insoluble proteins were removed by repeated dialysis and centrifugation. From the remaining soluble proteins, a LMW protein doublet was isolated by Sephadex G50 column chromatography and reversed-phase high-performance liquid chromatography (HPLC). The amino acid compositions of the 8 and 10 kDa proteins were similar to, but different from, those of known structural proteins in the stratum corneum. Rabbit antibody was raised against the 10-kDa protein band and cross-reacted with the 8-kDa protein band by Western immunoblot technique. The antibody recognized only this protein doublet amongst the proteins extracted from cultured keratinocytes. The 8- and 10-kDa proteins therefore appear to be closely related to, but different from, known structural proteins or degradation products. Immunofluorescence and immunoelectron microscopy demonstrated that this protein doublet was located in the cytoplasm of the cells of the malpighian layer, and in the outer layer of the marginal band of the horny layer. Its distribution was shown to be limited to stratified squamous epithelia by organ specificity studies. These results suggest that this protein doublet appears to be a novel soluble protein and a new epidermal diffferentiation marker of stratified epithelium.

The stratum corneum is formed by terminally differentiated layers of keratinocytes composed of soluble and insoluble proteins.[1] Cross-linked involucrin or keratolinin and other substrates form an insoluble and resistant marginal band or cornified envelope.[2–5] Polymerized keratins in filamentous formation can be solubilized only by denaturing conditions.[6] Filaggrin, a major component of the interfilamentous matrix, is readily solubilized.[7] Numerous known and unknown proteins of the stratum corneum are solubilized by water or buffer.

In a previous study we attempted to divide the human stratum corneum into three crude fractions: 8 M urea-2-mercaptoethanol-insoluble proteins (mainly marginal bands), 8 M urea-2-mercaptoethanol-soluble and repolymerized protein (keratin fibrous protein) and water-soluble proteins.[8] Fractionated crude proteins of stratum corneum samples, taken from normal healthy individuals and patients with a variety of keratinization disorders, were compared by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) or amino acid analysis. The water-soluble fraction of stratum corneum contained numerous unidentifiable proteins and polypeptides on SDS–PAGE analysis. Most polypeptide bands of soluble fractions vary from one individual to another in amount and molecular weight. It is, however, of interest that two strongly stained polypeptide bands of low molecular weights (8 and 10 kDa) are always present whether the skin is normal or affected by a pathological condition such as a disorder of keratinization. The abundance and invariable presence of this lower molecular weight (LMW) protein doublet in

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Correspondence: Professor Hideoki Ogawa.

* Present address: Department of Dermatology, School of Medicine, Chungnam National University, 640 Dasea-Dong, Chung-Ku, Daejon 300, Chungnam, Korea.

soluble protein from the stratum corneum suggests that it may have a significant biological function during keratinization.

In this study, we attempted to isolate and characterize this major LMW protein doublet from the soluble fraction of human stratum corneum.

Methods

Isolation of LMW (8 and 10 kDa) protein doublet

Plantar callus scraped from normal healthy volunteers was minced to a size approximately 1 mm^3 or less. Ten grams (dry weight) of the sample was placed in 500 ml of 8 M urea, 0·05 M Tris HCl, pH 9·0, 0·025 M 2-mercaptoethanol, stirred for 3 h at 20°C, and then ground in a tissue homogenizer. Cellular debris was removed by centrifugation at 30,000 *g* for 30 min. The extract was dialysed against distilled water for 24 h at 4°C and centrifuged at 250,000 *g* for 2 h. After removal of pellets (keratin fibrous fraction), the resultant supernatant was repeatedly dialysed against distilled water and centrifuged in the same manner until visible insoluble aggregates no longer appeared. The final supernatant (soluble fraction) was then lyophilized. Fifty-three milligrams of soluble fraction in 1 ml of 0·02 M phosphate buffer, pH 7·2, containing 0·1 M NaCl was applied to a 1·5 × 50 cm^2 Sephadex G 50 (Pharmacia, Inc. Piscatsway, NJ, U.S.A.) column equilibrated in the same buffer. Samples were eluted at a flow rate of 10 ml/h, and 2·3-ml fractions were collected. LMW protein doublet-rich fractions, which were identified by SDS–PAGE analysis, were collected and applied to reversed-phase high-performance liquid chromatography (HPLC) [TSK phenyl 5 pwRP, Tosoh, Tokyo] and eluted in an acetonitrile (Pierce Chemical Co., Rockford, IL, U.S.A.) gradient of 0–80% containing 1% trifluoroacetic acid (Pierce Chemical Co.).[9]

Preparation of antibody against 10-kDa protein

Following SDS–PAGE analysis, 10-kDa bands were cut out of gels stained with Coomassie Brilliant Blue R-250 (Nakarai Co., Tokyo, Japan). A 2-month-old female Japanese rabbit was immunized by intraperitoneal injection of these gel bands with an equal volume of Freund's complete adjuvant (Iatron Co., Tokyo, Japan). Four weeks later, the rabbit received two additional intraperitoneal injections with Freund's incomplete adjuvant (Iatron) at 2-weekly intervals. The control serum was collected from the rabbit before immunization.

Keratinocyte culture and extraction of proteins

Primary keratinocyte cultures were initiated from neonatal foreskins using the method of Reinwald and Green[10] in six-well plates. When attaining confluence, cells were collected from one of the wells and extracted with 0·5 ml of sample buffer for SDS–PAGE at 95°C for 5 min.

Amino acid analysis

Each band of 8- and 10-kDa protein was cut out of the gel after SDS–PAGE and Coomassie Blue staining. They were then minced in distilled water and centrifuged at 700 *g* for 30 min at 4°C. Extracts were dialysed against distilled water, lyophilized, and hydrolysed for 24 h in 6 N HCl at 110°C. Composition was determined on a Hitachi amino acid analyser (Model 835, Hitachi, Tokyo).

Indirect immunofluorescence procedure

Normal adult human skin and other human organs obtained at autopsy were subjected to immunofluorescence. Frozen tissues were cut into 8-μm sections, air-dried on glass slides, overlaid with serum from the immunized rabbit (1:200 dilution) for 30 min and then washed with phosphate-buffered saline (PBS). Sections were subsequently treated with affinity-purified fluorescein-conjugated goat anti-rabbit IgG (1:20 dilution, Cappel Laboratories, Cochranville, PA, U.S.A.) for 40 min and washed with PBS. The slides were then mounted in 90% glycerol-PBS and examined under a fluorescence microscope.

Immunoelectron microscopy

This was performed using an immunoperoxidase procedure.[11,12] Rabbit antibody against 10-kDa protein was used as a primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) as a second antibody. Pre-immunized serum was used as a control.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis. (SDS–PAGE) and Western immunoblots

Proteins were separated by SDS–PAGE,[13] transferred

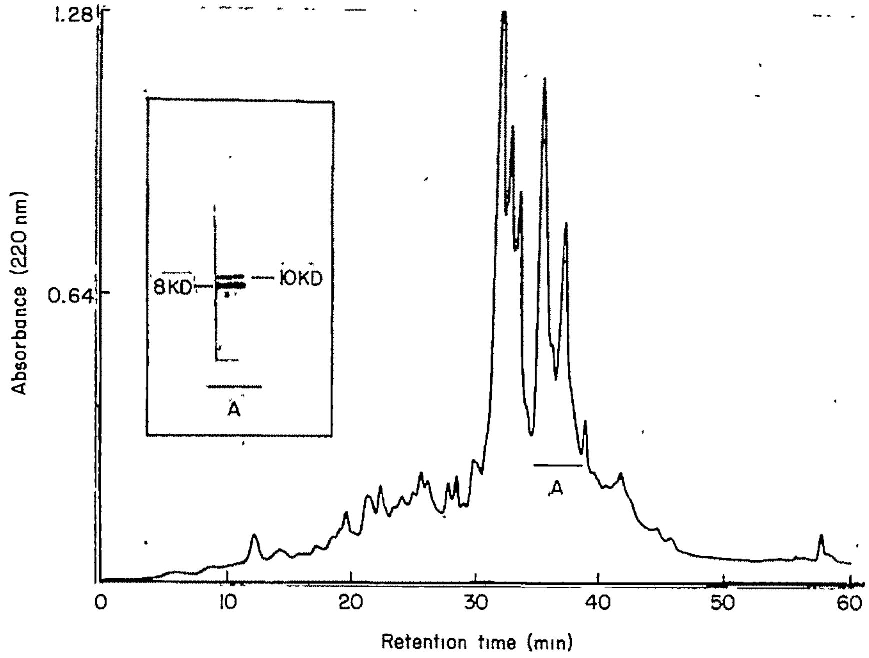

Figure 1. Elution profile of reversed-phase HPLC and SDS–PAGE of the peak A.

electrophoretically to nitrocellulose paper (Bio Rad, Richmond, CA, U.S.A.) by the method of Towbin *et al.*[14] and reacted with rabbit serum to 10-kDa protein or control serum. They were then developed with peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) followed by development with 0·0125% diaminobenzidine tetrachloride (Bio Rad) and 0·025% hydrogen peroxide.

Results

Isolation of 8- and 10-kDa protein doublet

Proteins obtained at each isolation step were evaluated by SDS–PAGE (Fig. 1). Numerous proteins, particularly keratins, were extracted by 8 M urea, Tris HCl, pH 9·0, containing 0·025 M 2-mercaptoethanol. After dialysis against water, most of the keratins and some higher

Table 1. Amino acid compositions of 8-kDa, 10-kDa, keratin, filaggrin, keratolinin, involucrin and marginal band

Amino acid	8 kDa	10 kDa	Keratin[15]	Filaggrin[7]	Keratolinin[3]	Involucrin[4]	Marginal band[5]
Aspartic acid	7·4	9·2	9·1	8·9	8·7	2·8	2·7
Threonine	4·4	4·6	3·3	1·2	10·5	1·6	3·1
Serine	16·2	14·6	13·2	13·8	9·2	1·6	13·6
Glutamic acid	11·0	13·1	14·1	14·8	13·5	45·8	11·1
Proline	2·5	3·1	0·9	0	4·5	5·7	13·3
Glycine	25·8	21·5	21·3	19·0	8·8	6·7	19·2
Alanine	8·5	6·9	4·5	6·6	8·5	1·5	3·5
Valine	4·1	4·6	3·4	4·3	6·3	3·7	4·1
1/2 cystine	Trace	Trace	1·1	Trace	1·0	—	10·5
Methionine	0·5	0	1·2	0·9	0·7	0·9	0·9
Isoleucine	2·7	3·1	3·6	2·3	3·2	0·4	1·4
Leucine	4·8	6·2	8·3	4·3	8·2	14·6	2·3
Tyrosine	1·4	0·8	2·6	2·1	1·7	0·8	2·4
Phenylalanine	2·2	2·3	3·0	1·9	2·7	0·6	2·6
Lysine	3·3	3·9	4·4	3·6	5·7	7·4	4·9
Histidine	2·2	1·5	0·9	6·5	1·2	4·7	2·0
Arginine	3·0	4·6	5·0	7·9	2·5	0·7	2·5
Ornithine	—	—	—	1·6	—	—	—
Citrulline	—	—	—	—	2·4	—	—
Tryptophane	—	—	—	—	0·7	0·2	—

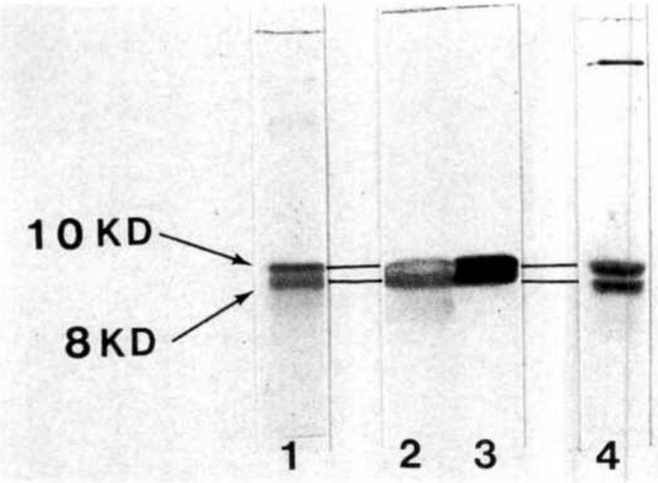

Figure 2. Western blot of the soluble fraction from urea 2-mercaptoethanol extracts (lane 2) and the direct PBS extract (lane 3) reacted with 1:200 dilution of rabbit anti-10-kDa protein serum. Lanes 1 and 4 show amido black stained soluble fraction (lane 1) and PBS extract (lane 4) transferred to nitrocellulose paper after running on SDS–PAGE.

molecular weight proteins were precipitated with the remaining supernatant (soluble fraction) which was mainly composed of lower molecular weight (less than 10 kDa) protein bands. The 8- and 10-kDa protein-rich fractions were collected by Sephadex G 50 gel filtration at an elution volume of 57·5–69·0 ml, although minimal amounts of higher molecular weight polypeptides were still present. Finally, the 8- and 10-kDa protein doublet was successfully obtained by collecting fractions at 35–40 min of retention time in reversed-phase HPLC (Fig. 1). The presence or absence of reducing agents during SDS–PAGE did not influence the migration of the doublet (data not shown).

Amino acid composition of 8- and 10-kDa proteins

The 8- and 10-kDa protein bands were cut from the SDS–PAGE gel and analysed for amino acid composition (Table 1). The amino acid compositions of the 8- and 10-kDa proteins were similar. In comparison with previously reported amino acid compositions of other well-known structural proteins of horny cells,[3–5,7,15] high contents of glycine, serine and glutamic acid were characteristic. These three amino acids comprised 49·2% and 53·0% of the total residues of the 8- and 10-kDa proteins, respectively. The amino acid composition of the 8- and 10-kDa proteins was apparently different from that of keratolinin, involucrin and marginal band. The composition also differed from keratin in having higher proline and lower 1/2 cystine content, and from filaggrin in having a lower histidine content.

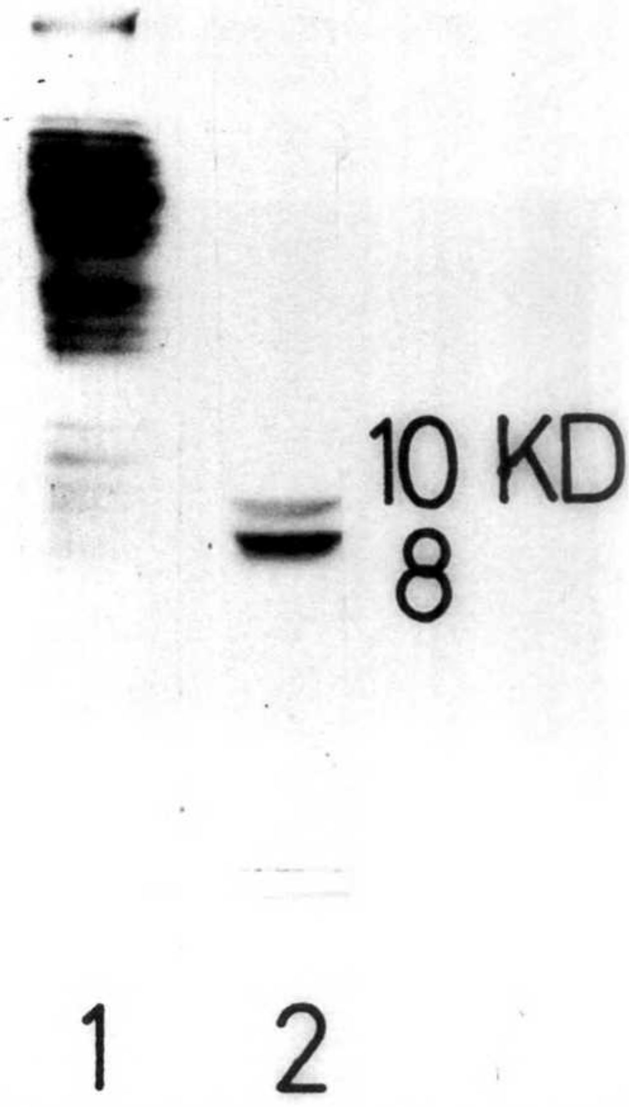

Figure 3. Western blot of cultured keratinocyte extracts, composed of mainly keratins and a lesser amount of lower molecular weight polypeptides (lane 1); reacted with 1:400 dilution of rabbit anti-10-kDa protein serum showed an immunoreactive staining at 8- and 10-kDa polypeptide bands (lane 2).

Western immunoblot analysis using anti-10-kDa protein antibody

Following immunization with 10-kDa protein, rabbit polyclonal antibody reactivity was examined by Western immunoblot. Of the proteins in the soluble fraction obtained by 8 M urea 0·025 M 2-mercaptoethanol extraction and dialysis (Fig. 2, lane 1), the antibody

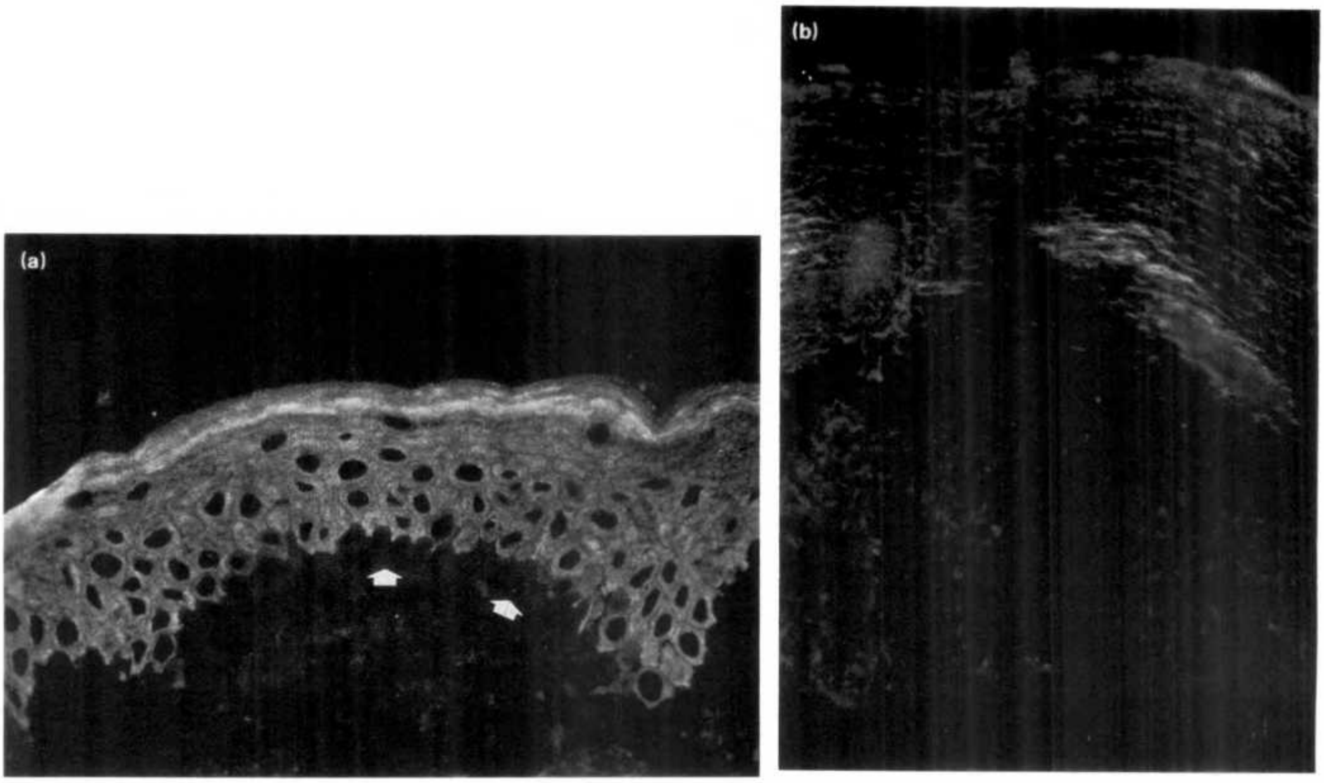

Figure 4. Indirect immunofluorescence with anti-10-kDa protein antibody using (a) body skin and (b) palmar skin. (a) The antigen localization shows a homogeneous diffuse cytoplasmic distribution in suprabasal living epidermal cells (arrows indicate epidermal–dermal junction). (b) There is linear distribution in the stratum corneum.

reacted not only with the 10-kDa protein but also cross-reacted with 8-kDa protein to the same intensity (Fig. 2, lane 2). When proteins were directly extracted by PBS and run on SDS–PAGE without procedures involving urea-reducing reagent extraction and dialysis, the molecular weights of the two major proteins were found to be identical with those in the soluble fraction (8 and 10 kDa) [Fig. 2, lane 4]. The anti-10-kDa antibody reacted with both of the proteins (Fig. 2, lane 3). Cultured keratinocyte extracts were mainly composed of keratins, with lesser amounts of lower molecular weight polypeptides (Fig. 3, lane 1). The antibody reacted with the proteins of 8 and 10 kDa molecular weight but not with keratins (Fig. 3, lane 2).

Localization of LMW protein doublet

Indirect immunofluorescence was carried out using body skin and palmar skin. Although the staining patterns in both specimens were essentially similar, better resolution was obtained in the living epidermis of body skin and in the stratum corneum of palmar skin. The anti-10 kDa protein antibody (anti-LMW protein doublet antibody) reacted with antigens in a homogeneous, diffuse cytoplasmic distribution in the suprabasal living epidermal cell layers (Fig. 4a). In contrast, there was a linear distribution of immunoreactive antigens in the stratum corneum, suggesting localization along horny cell membranes (Fig. 4b).

An immunoelectron microscopic study for ultrastructural localization of the 8- and 10-kDa protein doublet in the stratum corneum revealed that immunoreactive deposits were located at the outer layer of the marginal band, particularly in the intercellular spaces (Fig. 5). They were also found diffusely inside the plasma membranes of living epidermal cells, but there were no connections with or linkages to specific structures.

Antigen distribution in other epithelial tissues was examined by indirect immunofluorescence. Among a series of epithelia, including urinary bladder, trachea, intestine, liver and kidney, this LMW protein doublet was demonstrated only in stratified squamous epithelia

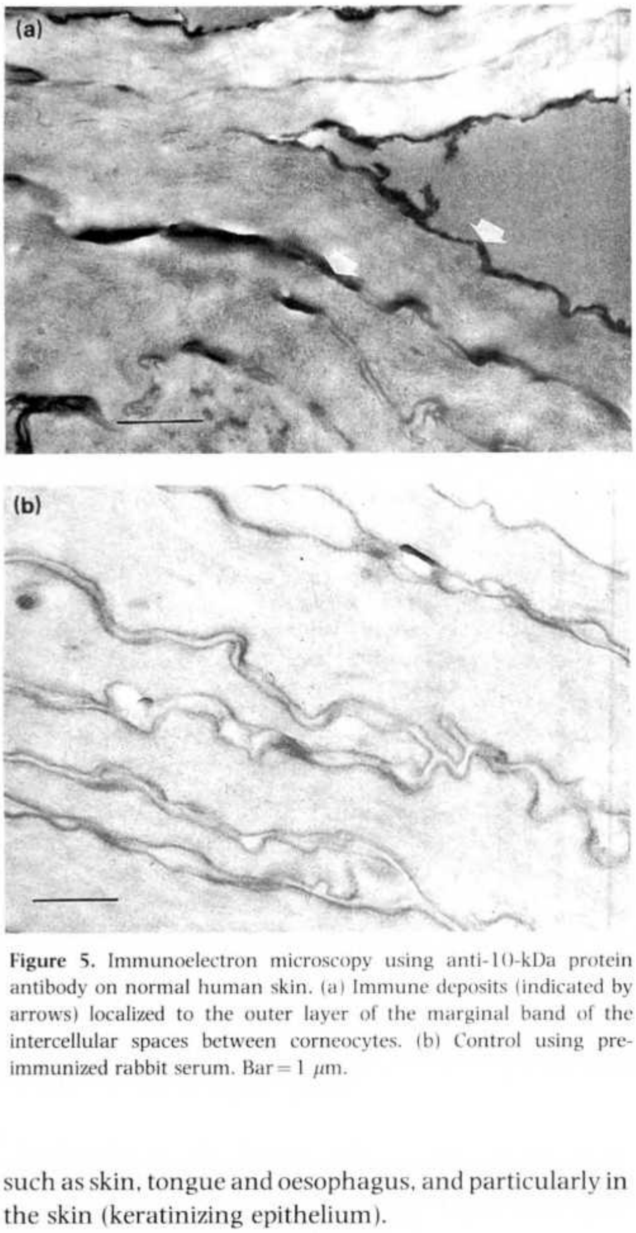

Figure 5. Immunoelectron microscopy using anti-10-kDa protein antibody on normal human skin. (a) Immune deposits (indicated by arrows) localized to the outer layer of the marginal band of the intercellular spaces between corneocytes. (b) Control using pre-immunized rabbit serum. Bar = 1 μm.

such as skin, tongue and oesophagus, and particularly in the skin (keratinizing epithelium).

Discussion

In the soluble fraction of human stratum corneum, the 8- and 10-kDa protein is found constantly and abundantly, no matter whether the source of the stratum corneum is normal or abnormal skin.[8] The proteins of this LMW doublet are closely related because of their similar amino acid composition, and also because they share immunogenicity. So far, chemical methods such as ion exchange, gel filtration, affinity chromatography and reversed-phase HPLC, have failed to separate this LMW doublet. The presence or absence of reducing agents during electrophoresis did not influence the migration of the doublet. It may be that the protein co-migrates as a doublet which contains minimal heterogeneity in the population of molecules.

When a direct PBS extract was used without vigorous chemical modification with urea and 2-mercaptoethanol, the antibody to the LMW protein doublet reacted with two polypeptides of molecular weights identical to those of 8 and 10 kDa in native form. It is unlikely that during the keratinization process the doublet is in a modified form such as degradation or cross-linking, because only 8- and 10-kDa bands out of the proteins in the cultured keratinocyte extract, with or without reduction, were recognized by the antibody.

The distribution of the LMW protein is unique. In the epidermis the doublet exhibited a sudden change in distribution from the cytoplasm of the living suprabasal epidermal cell layers to the intercellular spaces in the stratum corneum. It was not expressed by simple epithelia or non-epithelial tissues, but by stratified squamous epithelium, non-keratinizing or keratinizing, with different reactivity. In view of its unique suprabasal distribution in the epidermis, with a sudden change of localization from living keratinocytes to corneocytes, and its specific expression in stratified squamous epithelia, especially the epidermis, it is thought that the LMW protein doublet may be a marker protein of epidermal differentiation. These results suggest that biosynthetic products are released intercellularly at the level of the stratum corneum and, therefore, the LMW protein doublet may play an important role in corneocyte adhesion.

It is unlikely that the doublet is derived from other well-known structural proteins and epidermal differentiation markers such as keratin,[16] filaggrin,[7] involucrin,[4] keratolinin,[3] and marginal band,[5] because of different amino acid composition, immunohistochemical staining and molecular weight. The molecular weights of the LMW protein doublet were invariably 8 and 10 kDa whenever it was denatured, in native form, in living keratinocytes, and with or without reduction, and differed from those of other well-known structural proteins previously reported.[3–5,7,15] The amino acid compositions of the doublet were similar to those of keratins and filaggrin. However, filaggrin is characteristically rich in histidine[7] and histochemically is distributed in keratohyalin granules; keratin localization in stratum corneum is cytoplasmic, and the antibody to LMW protein did not react with keratins by immunoblot. Localization of the LMW protein was similar to that of marginal band precursor proteins such as keratolinin,[3] involucrin[4] and pancornulins.[17] The intracellular localization of keratolinin changes progressively from the cytoplasm in the basal layer to the cell membrane in the upper granular cell layer and stratum corneum.[3] Involucrin is present in the cytoplasm of the outer half of the spinous cell layer, but is concentrated peripherally long before cross-linking takes place at the site of stratum corneum formation.[4] However, the amino acid compositions of these transglutaminase substrates are quite different from those of the LMW protein doublet. Pancornulins are a group of low molecular weight proteins detected and characterized by monoclonal antibody raised against cornified envelopes.[17] Their distribution pattern differs from that of the LMW protein. Moreover, their molecular weights vary depending upon the culture state, which is not the case with the LMW protein doublet.

Non-ionic detergent-soluble proteins described by King *et al.*[18] may be closely related to the LMW protein because of similarities in molecular weights and distribution. Since these proteins are so crude that they reveal approximately 15 components in SDS-PAGE studies, the LMW protein doublet may possibly be derived from them. Other low molecular weight species found in the epidermis and stratum corneum such as interleukin 1,[19] lectin[20] or lysozyme[21,22] should be considered as having a possible relationship to the LMW protein, although molecular weights and distribution differ from those of our protein.

Studies such as 2-dimensional electrophoresis and specific bioassays would be necessary to establish that this LMW protein is a unique and new marker protein of stratified squamous epithelial differentiation.

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British Journal of Dermatology (1992) 127, 379–381.

Sensitivity of different areas of the flexor aspect of the human forearm to corticosteroid-induced skin blanching

E.MEYER,* E.W.SMITH AND J.M.HAIGH
School of Pharmaceutical Sciences, Rhodes University, Grahamstown, 6140 South Africa

Accepted for publication 4 June 1992

Summary

The intensity of corticosteroid-induced blanching has been found to vary at different areas of the flexor aspect of the human forearm. A retrospective analysis of 38,880 observations of skin blanching in 56 volunteers was conducted to assess the sensitivity of forearm skin to betamethasone 17-valerate. The mid-forearm appears to be more sensitive to the blanching response than do the areas close to the wrist or elbow. These results indicate that each preparation under evaluation should be applied to several sites along the forearm when using the human skin blanching assay in order to obtain an accurate comparative assessment of corticosteroid release from topical delivery vehicles.

Introduction

The human skin blanching (vasoconstriction) assay, first described in 1962[1] has been used extensively for 30 years as a method of assessing topical corticosteroid activity. This bioassay utilizes the skin whitening side-effect following topical application of corticosteroids to estimate the rate and extent of drug permeation through the stratum corneum. The intensity of induced blanching has been shown to correlate directly with the clinical efficacy of the steroid formulation.[2] Although the flexor aspect of the forearm is commonly used for blanching studies,[3,4] several comments have appeared in the literature concerning variations of the blanching response at different sites along the forearm. Blanching has been reported to vary from one site to another on the forearm after percutaneous absorption through normal or stripped skin,[5] to be poor at sites within about 4 cm of the wrist and elbow,[6] invariably to be more intense on the upper forearm than at the wrist[7] and to be inconsistent on persons with very short or narrow forearms, as well as in persons such as typists who use the forearm muscles consistently throughout the duration of corticosteroid application.[8] McKenzie and Atkinson[9] analysed the combined results of 29 blanching assays and found that there were highly significant differences between sites on the forearm, but reported no further details.

Kirsch *et al.*[10] were the first to attempt to quantify the sensitivity of various sites on the forearm to corticosteroid-induced blanching, and concluded that a gradient exists along the forearm, with the area close to the wrist showing less blanching than the area close to the elbow. This experiment was performed using occlusion, observations were made at only one reading time, and the trial was mounted to specifically assess this variable of the blanching assay.

Experience in our laboratories has indicated that blanching is not always consistent along the forearm, however, the existence of a gradient had not been noted. It was, therefore, decided to re-analyse data from previously performed trials in an attempt to ascertain whether such a gradient exists.

Subjects and methods

The methodology of the human skin blanching assay has been described in detail elsewhere.[3,4] An important difference between the methodology reported by Kirsch *et al.*[10] and that employed in this study is that the trials performed here were not designed to assess a possible gradient, that is to say, retrospective analyses were performed in order to obviate any possible subjectivity. In addition, observations were made at 10 reading times as opposed to one[11] and all the trials were performed in the unoccluded mode of application, the situation most often encountered clinically.

The blanching responses observed in three unoccluded trials were re-analysed. Two different proprietary cream formulations containing 0.12% betamethasone

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Correspondence: Dr J.M.Haigh.

*Present address: Pharmacy Department, The University of Sydney, Sydney, Australia.

17-valerate (Betnovate®, Glaxo, South Africa and Celestoderm-V®, Scherag, South Africa) were applied to 12 discrete sites on both forearms of 18 volunteers in each of the three trials. For the purpose of the re-analysis, the 12 sites on each arm were grouped into six pairs of adjacent sites (Fig. 1) which were termed wrist (W), wrist-1 (W-1), wrist-2 (W-2), elbow-2 (E-2), elbow-1 (E-1) and elbow (E). Each site-pair was represented by the two preparations. Four different application charts were used to prevent the emergence of a recognizable pattern during the observation procedure. The intensity of the induced skin blanching was independently assigned a 0, 1, 2, 3 or 4 ranking[3] by three trained observers at each observation time. Each set of results recorded at 7, 8, 9, 10, 12, 14, 16, 18, 28 and 32 h after application, for both arms of the 18 volunteers, for all three trials, were pooled for analysis. A total of 38,880 sites were observed, allowing the comparison of 19,440 site-pairs for the retrospective analysis. The significance of the differences in blanching between site-pairs was statistically assessed in two ways. As the observations of blanching intensity are subjective in nature, it is statistically more correct to assess this data in a qualitative manner. The χ^2-test may adequately be applied to assess the significance of the difference between this enumeration data. Alternatively, if the observed data is considered to be numerical (justified by the large number of observations in terms of the Central Limit Theorem) then the significance of the difference between the data obtained from different site-pairs may be evaluated using the *t*-distribution test (null-hypothesis of equal means, variance unknown[12]).

Results

Figure 1 depicts the average blanching score recorded at each of the six application sites along the forearm, at each of the 10 reading times. It is evident from this Figure that blanching appears to be maximal at sites in the central area of the forearm, at most observation times. Blanching is obviously of much lower intensity at sites close to the wrist and, to a lesser extent, at sites close to the elbow. This trend is clearly different from that reported previously[10] where a blanching intensity gradient decreasing from elbow to wrist was observed. However, direct comparison of the results is not possible

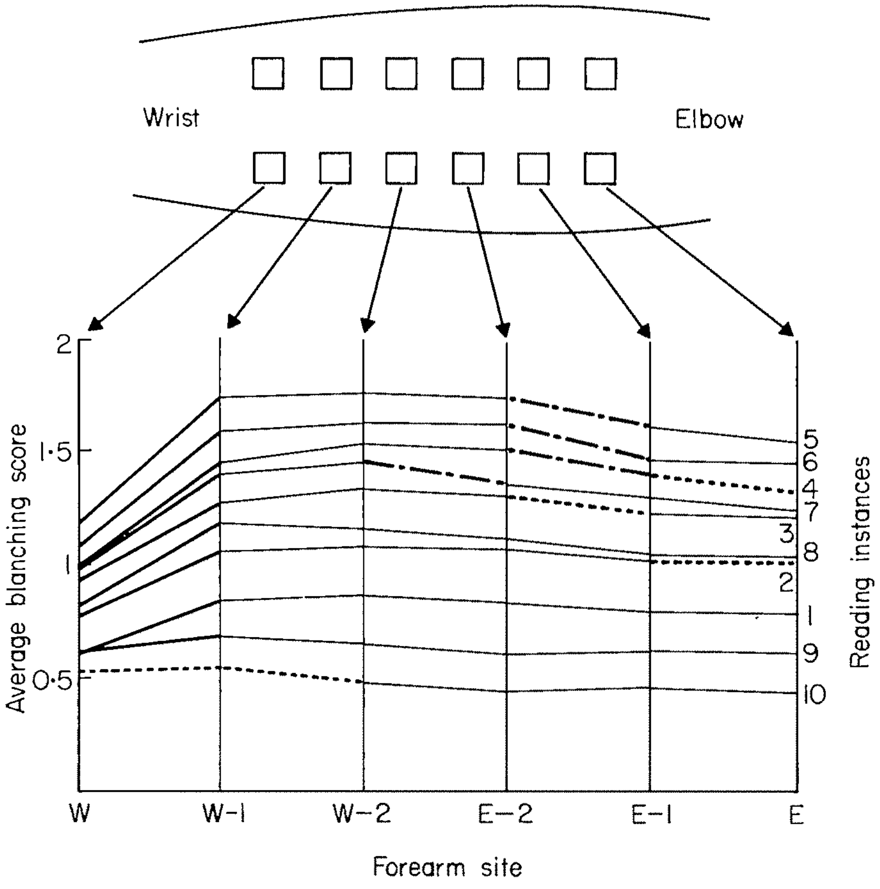

Figure 1. Average blanching score for demarcated forearm sites at 10 reading times. Statistically significant differences between adjacent sites obtained from both χ^2 test and *t*-test (——), χ^2 test (· · · ·) and *t*-test (·———·) are indicated.

since the experiment of Kirsch *et al.*[10] was performed in the occluded application mode and the recording of skin blanching was made at only one time after application, thereby producing fewer (120) observations for statistical analysis.

Statistical analysis (at the 95% level of significance) of the blanching intensity of adjacent site-pairs depicted in Figure 1 by both the χ^2- and *t*-test yields similar results. Both tests indicate statistically significant differences between the skin blanching intensity observed at the two sites closest to the wrist (W and W-1) at most reading times. There are also some calculated significant differences between site-pairs along the forearm. It should be noted that statistical analysis of data from non-adjacent sites is not depicted in Figure 1, hence at most reading times there are also significant differences between the scored intensity at, for example, the wrist (W) and position wrist-2 (W-2).

Discussion

The human skin blanching assay is used to assess the topical availability of corticosteroids from various delivery systems. Usually, 12 application sites per forearm are demarcated for use. Given that blanching inconsistencies do occur along the human forearm, we strongly recommend that each preparation under test should be applied to several sites along the forearm of each volunteer in order to obtain an accurate comparative assessment of corticosteroid release from topical delivery vehicles. It is clear from the above results that comparisons of blanching intensity between a formulation applied close to the wrist and one applied to the mid-forearm would result in erroneous conclusions due to this physiologically inconsistent response.

Acknowledgments

The authors gratefully acknowledge financial assistance received from the Foundation for Research Development and the Rhodes University Council.

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British Journal of Dermatology (1992) 127, 382–386.

Inhibition of human monocyte functions by anthralin

U.MROWIETZ, M.FALSAFI, J.-M.SCHRÖDER AND E.CHRISTOPHERS
Department of Dermatology, University of Kiel, Kiel, Germany

Accepted for publication 4 June 1992

Summary

Anthralin is a well-established and widely used compound for topical treatment of psoriasis. In recent years attention has been focused on the anti-inflammatory properties of anthralin, with particular reference to psoriasis. In this study the effect of anthralin on human monocyte chemotaxis, superoxide-anion generation, and enzyme degranulation, were investigated. For comparison, the effect of the clinically inactive anthralin derivative danthrone and the solvent (acetone) were also studied. The results show that anthralin potently inhibits stimulated human monocyte superoxide-anion generation and enzyme degranulation, with a half-maximal inhibitory concentration (IC_{50}) of as low as 0·02 μg/ml. Chemotactic migration of monocytes, however, was only affected when very high doses of anthralin (10 μg/ml) were used for pretreatment of the cells. Danthrone, up to a concentration of 10 μg/ml, or acetone alone (0·1%, v/v), did not inhibit the monocyte functions tested. Our results indicate that anthralin at pharmacological concentrations is a potent and selective inhibitor of human monocyte pro-inflammatory activities, by inhibiting respiratory burst activity (e.g. superoxide-anion generation) and enzyme degranulation, without affecting chemotactic migration.

Anthralin has become a commonly used topical antipsoriatic treatment since it was introduced by Galewsky and Unna in 1916.[1,2] During the past few decades numerous clinical and experimental studies have been performed which have focused on the mode of action of anthralin in the skin. In recent years a number of anti-inflammatory properties of anthralin of relevance in psoriasis have been elucidated.[3]

Previous work from our laboratory revealed a dose-dependent inhibition of human neutrophil granulocyte functions by anthralin, including chemotaxis and superoxide-anion generation, whereas the stimulated release of neutrophil granule enzymes was not affected.[4] The clinically inactive derivatives danthrone and dimeric anthralin did not show any inhibitory activity in these systems. In a subsequent study it was demonstrated that anthralin potently inhibits leukotriene production and leukotriene B_4-ω-oxidation by human neutrophils.[5]

In view of the complexity of the psoriatic tissue reaction, it is important to examine inflammatory cells other than those studied to date. Enhanced functional activities in monocytes have been reported in patients with psoriasis,[6,7] and the early appearance of these cells in a newly evolving psoriatic plaque has also been described.[8] In addition the presence of these cells in the dermal perivascular mononuclear cell infiltrate suggests a possible pathogenic role in this disease.[9]

In this study we investigated the effect of anthralin and its clinically inactive oxidation product danthrone on human monocyte functions. Chemotactic migration, superoxide-anion generation and enzyme degranulation were measured after pre-incubation of the cells with various concentrations of anthralin, danthrone, buffer or solvent. Our results suggest that monocyte superoxide-anion release and enzyme degranulation are sensitive targets for the inhibitory effect of anthralin, whereas chemotactic migration of these cells was only slightly decreased.

Methods

Chemicals

Anthralin (1,8-dihydroxyanthrone) and danthrone (1,8-dihydroxydianthrone) were purchased from Sigma (Munich, Germany). Both substances were dissolved in reagent-grade acetone (Merck, Darmstadt, Germany) to 10 mg/ml, and further dissolved in phosphate-buffered saline (PBS) containing 0·1% bovine serum albumin (w/v) (Sigma), 0·1% glucose (w/v), 0·9 mM $CaCl_2$, and 0·5 mM $MgCl_2$ (referred to as 'complete buffer').

Correspondence: Dr Ulrich Mrowietz, Department of Dermatology, University of Kiel, Schittenhelmstr. 7, D-2300 Kiel 1, Germany

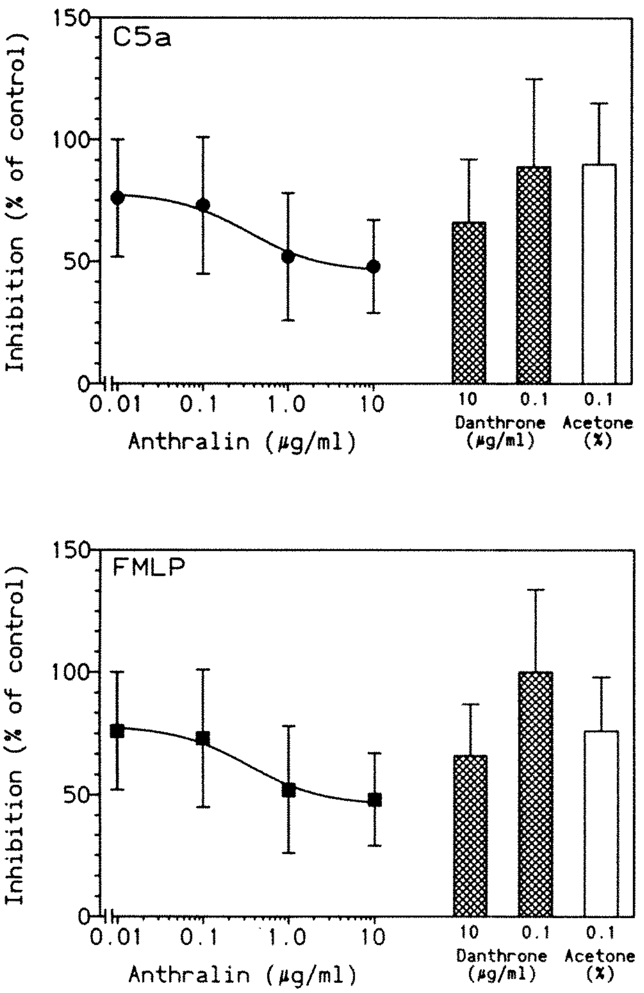

Figure 1. Human monocyte chemotaxis in response to C5a 1 : 5 pool-dilution [●—●] and FMLP 10^{-8} M [■—■] after pretreatment of the cells with anthralin (0·01–10 µg/ml), danthrone (0·1 and 10 µg/ml) [▨] and acetone (0·1%, v/v) [□]. Results are expressed as percentage of the buffer-treated control. Only slight inhibition of chemotaxis by anthralin can be seen. Mean and SD, $n = 6$.

Stimuli

N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma) was dissolved in DMSO to a stock concentration of 10^{-2} M. Complement split-product 5a (C5a) was purified according to a modification of the method of Fernandez and Hugli,[10] as described,[11] and dissolved in 10^{-3} M HCl. Opsonized zymosan particles (C3b-zymosan) were prepared by incubation of boiled zymosan A (Sigma) in pooled fresh human serum for 2 h at 37°C. After repeated washing with PBS, opsonized zymosan particles were suspended in 'complete buffer' at a concentration of 2·5 mg/ml. Phorbol-myristate-acetate (PMA) (Sigma) was dissolved in DMSO to a stock concentration of 1 mg/ml. All prepared reagents were

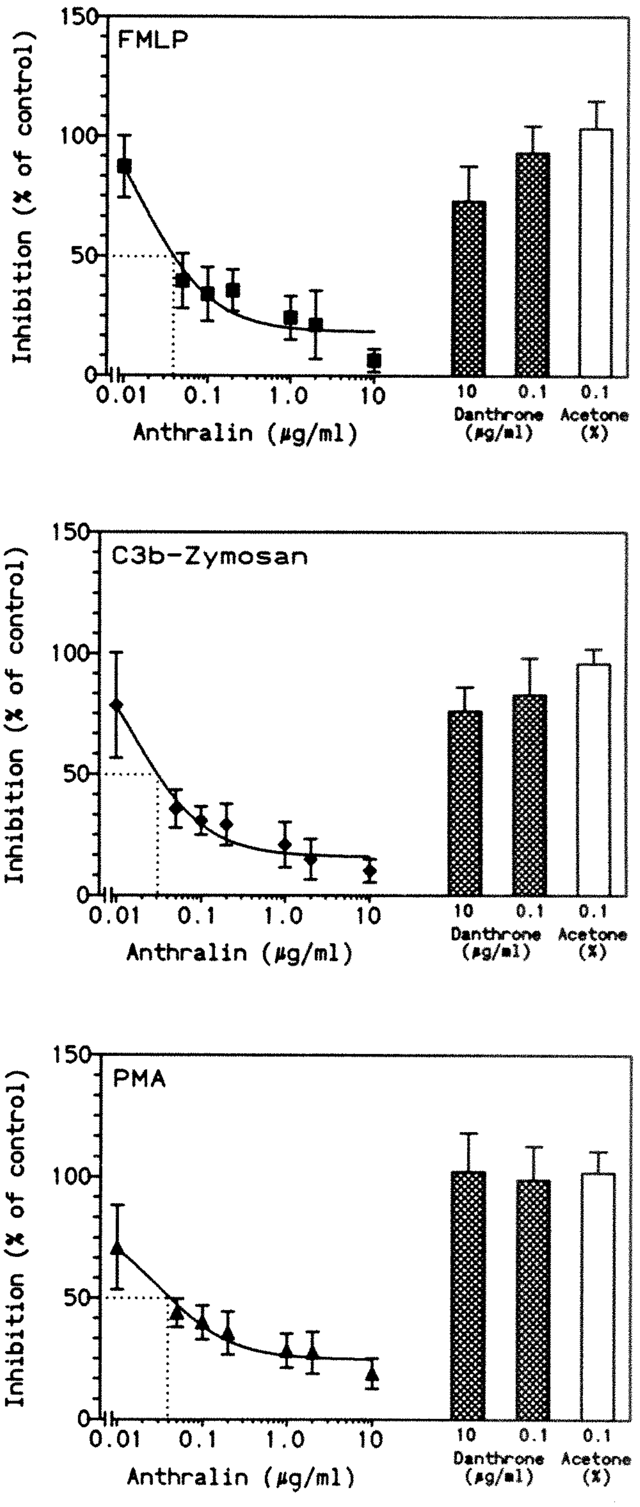

Figure 2. Inhibition of human monocyte superoxide-anion production in response to FMLP (10^{-7} M) [■—■], opsonized zymosan (250 µg/ml) [◆—◆] and PMA (1 ng/ml) [▲—▲] after anthralin (0·01 to 10 µg/ml), danthrone (0·1 and 10 µg/ml) [▨] and acetone (0·1%, v/v) [□] pretreatment. Results are expressed as percentage of the buffer-treated control. Mean and SD, $n = 7$.

stored in aliquots at −70°C and diluted to the appropriate concentrations with 'complete buffer' before use.

Monocyte isolation

Purified human monocytes were prepared as described previously.[12] Briefly, venous blood from healthy volunteers was anticoagulated with EDTA, underlayered by Ficoll-separation medium (Biochrom, Berlin, Germany) and centrifuged for 35 min. at 400 *g* at 20°C. Thereafter the interphase was harvested and monocytes were separated from lymphocytes by their receptor-mediated reversible attachment to gelatin-bound fibronectin. Isolated monocytes were 92 ± 4% pure by morphology and non-specific esterase staining. Viability was 98% by trypan blue dye exclusion.

Pretreatment of monocytes

Monocytes were pre-incubated with anthralin (0·01–10 μg/ml), danthrone (0·1 and 10 μg/ml) or acetone (final concentration 0·1%, v/v) for 30 min at 37°C. Thereafter cells were washed twice and finally resuspended in 'complete buffer'.

Chemotaxis assay

Monocyte chemotaxis and random migration were measured using Boyden blind-well chambers employing a double membrane/filter technique (upper 5·0 μm pore-size PVP-free polycarbonate membrane, lower 0·45 μm cellulosic filter, Nuclepore, Tübingen, Germany) as described elsewhere.[12] Chambers were incubated for 30 min at 37°C, and migrated cells adhering to the lower filter were quantified indirectly using lactic dehydrogenase as a marker enzyme. Chemotactic migration was elicited by FMLP (10^{-8} M) and C5a (1 : 5 pool-dilution). Random migration was assessed using 'complete buffer' instead of the stimulus, in the same system.

Superoxide-anion generation

Superoxide-anion (O_2^-) generation was determined in the cytochrome *c* system according to a modification of the method of English *et al.*,[13] as described elsewhere.[14] Monocytes pre-incubated with cytochalasin *b* (5 μg/ml for 5 min at 37°C) were further incubated for 20 min at 37°C together with stimuli (FMLP 10^{-7} M, C3b-zymosan 250 μg/ml or PMA 1 ng/ml) and cytochrome *c*. After rapid cooling the amount of reduced cytochrome *c* was measured photometrically at 550 and 474 nm vs. standards of oxidized and totally reduced (sodium dithionite) cytochrome *c*.

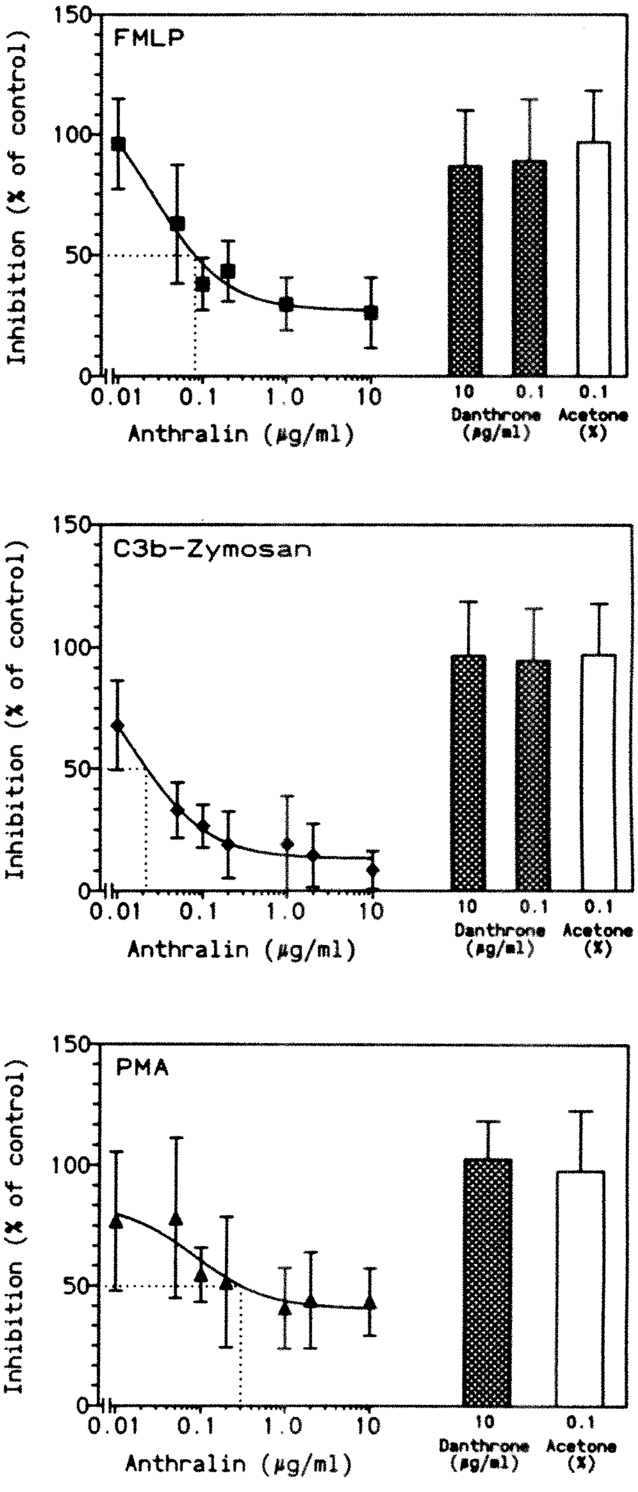

Figure 3. Inhibition of human monocyte β-glucuronidase release in response to FMLP (10^{-7}) [■—■], opsonized zymosan (250 μg/ml) [◆—◆] and PMA (1 ng/ml) [▲—▲] after pretreatment of the cells with anthralin (0·01–10 μg/ml), danthrone (0·1 and 10 μg/ml) [▨] and acetone (0·1%, v/v) [□]. Results are expressed as percentage of the buffer-treated control. Mean and SD, $n = 7$.

β-glucuronidase release

The stimulated release of β-glucuronidase (β-glu) from monocytes was measured, as previously described, using *p*-nitrophenyl-β-D-glucuronide as substrate.[14] Monocytes were pre-incubated with cytochalasin *b* (5 µg/ml) for 5 min at 37°C and subsequently stimulated with FMLP 10^{-7} M, C3b-zymosan 250 µg/ml or PMA 1 ng/ml) for 30 min at 37°C. Thereafter the substrate (0·01 M in 0·1 M sodium acetate, pH 4) was added to the cell-free supernatant, followed by incubation for 18 h at 37°C. After incubation, glycine buffer (0·4 M, pH 10) was added, and the yellow-coloured complex was measured in a photometer at 404 and 620 nm. A 100% control was obtained by addition of triton X-100 (0·1%, v/v) to a cell-containing sample. Enzyme release was expressed as a percentage of the 100% control.

Statistical analysis

Mean and SD were calculated from values obtained. Results of the anthralin-, danthrone- or acetone-treated samples were calculated as a percentage of the buffer-treated control. Curves shown in Figures 1, 2 and 3 were plotted after non-linear regression analysis.

Results

Monocyte viability

After pretreatment with anthralin (up to 10 µg/ml), danthrone (up to 10 µg/ml) or acetone (0·1%, v/v) monocyte viability exceeded 95% as assessed by trypan blue dye exclusion.

Chemotaxis

The results showed that anthralin slightly inhibited monocyte chemotaxis in response to C5a and FMLP. A 50% inhibition, however, could be achieved by anthralin concentrations as high as 10 µg/ml (Fig. 1). As indicated in Table 1, monocyte random migration was affected to a similar degree by anthralin.

Danthrone (up to 10 µg/ml) and acetone (0·1%, v/v, corresponding to the highest anthralin concentration) had no significant effect on monocyte chemotaxis and random migration (Fig. 1 and Table 1).

Superoxide-anion generation

Dose-dependent inhibition of O_2^--generation from monocytes after pretreatment with anthralin, danthrone or acetone in response to C3b-zymosan, FMLP and PMA is demonstrated in Figure 2. A 50% inhibition of O_2^--production was seen in a dose range of anthralin between 0·02 and 0·05 µg/ml for all three stimuli. Danthrone and acetone (corresponding to 10 µg/ml anthralin) did not show a significant inhibitory effect (Fig. 2).

Table 1. Effect of anthralin on human monocyte random migration

	Anthralin (µg/ml)				Danthrone (µg/ml)		Acetone 0·1% (v/v)
	0·01	0·1	1·0	10	0·1	10	
Inhibition	70	82	66	46	58	89	71
(% of buffer control)	±37	±59	±34	±19	±25	±48	±23

Mean and SD, $n=6$.

β-glucuronidase release

Inhibition of β-glucuronidase release from anthralin, danthrone or acetone pretreated monocytes in response to C3b-zymosan, FMLP or PMA is shown in Figure 3. For anthralin a dose-dependent inhibition of monocyte β-glucuronidase release was observed, with a 50% inhibition in a dose range of 0·02 and 0·08 µg/ml anthralin for C3b-zymosan and FMLP stimulation, respectively. For PMA, 50% inhibition was achieved at an anthralin concentration of 0·2 µg/ml.

Danthrone or acetone did not inhibit stimulated β-glucuronidase release from monocytes (Fig. 3).

Discussion

Our results indicate that anthralin is able to inhibit human monocyte superoxide-anion production and enzyme degranulation in a dose-dependent manner. On the other hand, monocyte random migration and chemotaxis in response to C5a and FMLP were not significantly decreased by various concentrations of anthralin. Danthrone, and the solvent acetone, did not influence the monocyte functions measured.

Schröder *et al.*[4] described inhibitory activity of anthralin on human neutrophil granulocyte functions. In their study anthralin, but not its clinically inactive derivatives danthrone and dimeric-anthralin, strongly inhibited neutrophil chemotaxis and superoxide-anion generation with an IC_{50} of about 0·5 to 1·0 µg/ml (Table 2). The stimulated release of the neutrophil enzyme β-glucuronidase was not inhibited by anthralin. In a subsequent study it was demonstrated that anthralin is a potent

Table 2. Inhibition of human leucocyte functions by anthralin as shown by IC_{50}. Comparison of the results with human monocytes obtained in this study, and neutrophils from the work of Schröder *et al.*[4]*

	Monocytes (μg/ml)	Neutrophils* (μg/ml)
Chemotaxis	NI	0·5
Enzyme-release	0·02–0·2	NI
O_2^--production	0·02–0·04	1·0

NI, no significant inhibition.

inhibitor of leukotriene B_4 formation and its ω-oxidation in stimulated human neutrophils.[5]

Ternowitz[7] showed that monocytes from psoriatic patients have increased chemotactic activity, which returns to normal after topical anthralin treatment.[15] Concentrations of anthralin inhibiting monocyte (and neutrophil) functions *in vitro* seem to be of relevance *in vivo*. Kammerau *et al.*[16] demonstrated that in a human *in vivo* model anthralin concentrations of 21·1 μg/ml, which are one hundred times the IC_{50} for leucocyte functional inhibition, could be achieved in the epidermis after application of 0·1% anthralin in white petrolatum for 5 h. Levels measured in the dermis were in the range of the observed IC_{50} for monocyte and neutrophil inhibition (0·2 μg/ml). Similar results were obtained in a subsequent study by Schalla *et al.*[17] using fresh cadaver skin. These evaluations were performed by separating epidermis and dermis prior to anthralin measurements, suggesting that, *in vivo*, a concentration gradient of the drug may lead to anthralin-concentrations at the dermal–epidermal junction far exceeding IC_{50} levels for leucocyte inhibition.

Our results provide further evidence that anthralin is a multifunctional inhibitor of human leucocyte proinflammatory activities. However, monocyte chemotaxis and, as reported earlier,[4] neutrophil β-glucuronidase release, were only slightly affected by anthralin. In both cells this observation was not restricted to one stimulus, which makes a receptor-related phenomenon unlikely.

In neutrophils and monocytes, production of superoxide anions is inhibited by anthralin. However, comparing IC_{50}, monocytes appeared about one hundredfold more sensitive to inhibition by anthralin than neutrophils. The reason for the higher susceptibility of monocyte functions to inhibition by anthralin in comparison with neutrophils cannot be explained at present. As it has been shown that anthralin occlusion leads to fine structural changes in human Langerhans cells, but not in keratinocytes,[18] a high sensitivity of cells of the monocyte/macrophage lineage to anthralin may be assumed. Subsequent studies addressing this issue may therefore be of importance.

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British Journal of Dermatology (1992) 127, 387–391.

Bone changes and their significance in children with ichthyosis on long-term etretinate therapy

D.G.PAIGE, M.R.JUDGE, D.G.SHAW,* D.J.ATHERTON AND J.I.HARPER
*Departments of Dermatology and *Radiology, The Hospital for Sick Children, Great Ormond Street, London WC1N 3JH, U.K.*

Accepted for publication 20 May 1992

Summary

Anxiety about the use of etretinate in children has been provoked by several reports describing skeletal abnormalities during long-term therapy. However, we have observed no evidence of skeletal toxicity in 42 children treated over an 11-year period. Radiological screening before and during treatment has failed to reveal abnormalities that would influence our decision to commence or to continue etretinate administration.

We recommend that children who are to be treated with etretinate should have a baseline selective skeletal survey, with follow-up radiology restricted to those with pretreatment radiological abnormalities and those who develop musculo-skeletal symptoms. In addition we advise that dosage should not exceed 1 mg/kg/day. If these guidelines are followed, we believe that long-term therapy with etretinate can be given to children, with an acceptable margin of safety.

Etretinate has proved to be a useful drug in the management of severe ichthyosis. Whilst drug-induced skeletal toxicity remains a concern, unnecessary exposure to ionizing radiation from frequent radiological investigations must also be considered. There are no generally accepted guidelines governing the long-term radiological follow-up of children who are on etretinate. We have reviewed our experience with long-term etretinate in an attempt to develop a rational and safe approach to this problem.

Patients and methods

A total of 42 children with congenital ichthyosis received oral etretinate in the period 1980 to 1991. Twenty-four were male and 18 were female. Their ages at the start of therapy ranged from 6 months to 16 years (mean 7·5 years). The duration of treatment ranged from 1 month to 11 years (mean 5 years). Starting doses ranged from 0·4 to 1 mg/kg/day. In no patient did the dosage at any time exceed 1 mg/kg/day.

The patients' diagnoses were as follows: 15 bullous ichthyosiform erythroderma (BIE), 14 non-bullous ichthyosiform erythroderma (NBIE), three lamellar ichthyosis (LI), four Sjögren–Larsson syndrome (SLS), three Netherton's syndrome (NS), and one each had unclassified psoriasiform ichthyosis, neutral lipid storage disease and trichothiodystrophy (IBIDS).

^{99}Technetium methylene diphosphonate(^{99m} TcMDP) whole body bone scanning with high-resolution collimation was used for monitoring changes in osteogenesis and abnormalities of the growth plate. Dosage was calculated according to body surface area, based on an adult dose of 444 mBq/1·73 m^2. Appropriate plain radiographs were carried out in those patients in whom scans indicated skeletal defects, and selective skeletal surveys (skull views excluded) were carried out in those patients who were considered unsuitable for scanning on technical grounds (e.g. patients with Sjögren–Larsson syndrome with spinal or leg deformities).

From 1984, following early reports of skeletal toxicity in patients on retinoid therapy, new patients receiving retinoids at our hospital were required to have pretreatment radiological investigations. All patients were subsequently followed up with biennial bone scans and plain radiographs of any abnormal areas. Patients were reviewed every 6 months, when clinical assessment and growth measurements were undertaken. Between scans, patients were advised to report any musculoskeletal symptoms, and these were appropriately investigated.

Nineteen patients had pretreatment investigations: 10 by isotope bone scan, nine by skeletal survey, and they were followed up prospectively.

Twenty-seven patients had skeletal investigations

Correspondence: Dr J.I.Harper, Consultant Dermatologist, The Hospital for Sick Children, Great Ormond Street, London WC1N 3JH, U.K.

during therapy. Fifteen patients did not, either because treatment duration was less than 2 years, or because treatment was stopped due to poor clinical response.

Results

The cumulative duration of etretinate treatment was 199 years. Fifty-four isotope bone scans and 12 skeletal surveys were reviewed. Growth measurements remained normal in all patients.

Baseline studies

The results of the baseline studies are shown in Table 1. Fifteen of the 19 patients, who were followed prospectively from the outset, had normal baseline studies. Four patients showed asymptomatic skeletal abnormalities prior to starting therapy. These radiological abnormalities were not considered to be a contraindication to starting etretinate therapy. Subsequent follow-up bone scans were normal in three of these patients; the fourth patient has been on treatment for only 1 year and is awaiting follow-up studies.

Follow-up investigations

The results of the follow-up investigations are shown in Table 2. Seven of the 27 patients were shown to have asymptomatic skeletal abnormalities on follow-up bone scans and radiographs. Of these seven, six started treatment prior to 1984 and no baseline studies were available for comparison. The radiological abnormalities were not regarded as clinically significant, so etretinate therapy was continued in all cases. The most recent bone scans were normal in four of these six cases. Of the other two patients, one is awaiting further follow-up studies and one has stopped etretinate therapy because of diffuse alopecia. The one patient who had a baseline study (normal bone scan), showed increased uptake in the right knee on a follow-up bone scan 5 years after starting therapy. A corresponding plain radiograph at that time was normal. He has remained asymptomatic, and treatment has continued at the same dose. He awaits further follow-up studies. The other 20 patients have had normal follow-up investigations.

Discussion

Vitamin A is essential for epithelial growth and differentiation. It is also necessary for normal bone metabolism. Systemic vitamin A therapy was introduced in the 1960s and used for severe congenital ichthyoses with variable, but sometimes dramatic, results. Such treatment was limited by severe toxicity, particularly neurological adverse affects. The skeletal side-effects noted were cortical hyperostosis, ligamentous and tendon calcification, and premature epiphyseal closure.[1–3] These changes were thought to be a result of accelerated bone and cartilage resorption, with new bone formation.[4]

Vitamin A therapy was superseded by development of the synthetic analogues isotretinoin and etretinate. During the 1980s it became clear that similar skeletal changes were sometimes seen in adults on long-term

Table 1. Radiological changes detected prior to starting etretinate therapy in four of 19 patients

No.	Age/sex	Diagnosis	Radiological abnormalities	Initial dose of etretinate (mg/kg/day)	Outcome
1	3/M	BIE	Bone scan showed increased uptake in the lower femoral and upper tibial metaphyses. Corresponding X-rays were normal	0·5	Bone scan 2 years later was normal. He continues on therapy (0·5 mg/kg/day)
2	4/F	NBIE	Bone scan showed increased uptake in the left 6th rib. X-ray of the area was normal	0·5	Bone scan 2 years later was normal. He continues on therapy (0·75 mg/kg/day)
3	3/M	NBIE	Skeletal survey showed generalized osteoporosis, slender long bones with multiple growth arrest lines. Biochemical studies normal	0·4	Bone scan at 2 years was normal with no defects in the growth plates. He continues on therapy (0·5 mg/kg/day)
4	2/M	NBIE	Skeletal survey showed generalized osteoporosis with irregularity of long bone metaphyses	0·5	On treatment for 1 year and awaiting follow-up studies

Table 2. Radiological changes detected during etretinate therapy in seven of 27 patients

No.	Age/sex	Diagnosis	Treatment	Radiological abnormalities	Outcome
1	15/M	BIE	1980—etretinate started at age 4 (1·0 mg/kg/day). Dose reduced to 0·75 mg/kg (1986) and 0·6 mg/kg (1988) with no clinical deterioration	1986 bone scan—increased uptake in tibiae but X-rays were normal. 1988 scan showed increased uptake in the right tibia and right 4th rib	Treatment continued 1989 and 1990 bone scans were normal
2	13/F	BIE	1984—etretinate started at age 6 (0·75 mg/kg/day) Dose reduced to 0·6 mg/kg (1988) with no clinical deterioration.	1986 bone scan—increased uptake in head of 5th rib (X-rays normal) 1988 scan normal 1990 scan—increased uptake in upper tibiae (X-rays normal)	Treatment continued Awaiting further follow-up
3	14/F	BIE	Previous vitamin A therapy 1980—etretinate started at age 4 (1 mg/kg/day)	1986 bone scan—increased uptake right hand & left knee but X-rays normal	Previous surgery to left knee noted. Etretinate continued until 1987 but stopped because of diffuse alopecia
4	15/F	NBIE	1981—etretinate started at age 5 (0·5 mg/kg/day). Dose has remained unaltered	1985 bone scan—increased uptake in medulla of both tibiae (X-rays normal)	Treatment continued 1988 and 1989 scans normal
5	15/M	BIE	1981—etretinate started at a dose of 0·8 mg/kg/day decreasing to maintenance dose of 0·5 mg/kg by 1984.	1986 bone scan—increased uptake in right 1st MTP joint (X-ray showed early osteoarthritic changes)	Treatment continued. 1988 scan no change, 1990 scan normal
6	11/F	LI	1981—etretinate started at age 2 (0·5 mg/kg/day). From 1984—maintenance dose of 0·7 mg/kg/day	1987 skeletal survey—small exostosis upper left tibia	Treatment continued. 1989 bone scan and tibial X-rays normal
7	16/M	BIE	1984—etretinate started at a dose of 0·75 mg/kg/day (3-month drug holidays each year in the summer)	Baseline and 1987 bone scans were normal. 1989 scan—increased uptake lateral condyle right knee (X-ray normal)	Treatment continued Awaiting further follow-up

isotretinoin[5–9] and etretinate therapy.[10–14] Skeletal abnormalities in children were first reported in 1985; changes including osteoporosis, periosteal thickening, ligamentous calcification and hyperostoses were observed in three out of four children receiving long-term isotretinoin.[9] Similar changes have been recorded less commonly with etretinate.[15,16] Although diffuse 'idiopathic' skeletal hyperostosis (DISH) has been reported in an adult on etretinate,[17] this has not been reported in a child to date. Symptomatic ossification of the interosseous membrane of the forearms has been described in three adults on long-term etretinate,[18–21] one of whom was 15 years of age at the commencement of treatment.[19] The most serious skeletal abnormality reported in children receiving oral retinoids is premature epiphyseal closure.[22,23] It has, however, been argued that closure may have been 'physiological' in the case of one girl, who had early menarche with advanced bone age.[24]

Some studies, however, suggested isotretinoin[25] and etretinate[26–30] were surprisingly well-tolerated in children. Tamayo *et al.* treated 30 children with etretinate for 16–24 months.[27] One of these children, with severe lamellar ichthyosis, suffered two traumatic fractures during therapy. However, slender long bones were noted at the time of X-ray and these changes may have preceded treatment, as no baseline films were available. Glover *et al.* reported 19 children on long-term etretinate therapy (median 5 years) who suffered no significant skeletal abnormalities.[29] The above studies and reports provide a somewhat conflicting picture of retinoid skeletal toxicity. We feel the following points are important in their interpretation.

A feature of many of the cases in which skeletal abnormalities have been reported has been relatively high dosage, often well in excess of 1 mg/kg/day.[7,10,16,22,28] Very few skeletal problems have been described in children receiving long-term dosage less than 1·0 mg/kg/day. Furthermore, several patients with skeletal abnormalities had previously been treated with vitamin A or isotretinoin, and this may have been an important contributory factor.[17,22,23]

Another problem has been the interpretation of radiological abnormalities in those patients who have not had baseline studies at the initiation of therapy. Trauma, surgery and the skin diseases themselves may have been responsible for pre-existing abnormalities; such changes have been reported both in psoriasis[7,31,32] and the ichthyoses[16,33] This makes the link between retinoids and bone changes difficult to interpret if pretreatment studies are not undertaken. Direct comparison of patient groups with different diagnoses is also not appropriate.

Further difficulties arise due to the different radiological techniques used for investigation. TcMDP whole body bone scanning is more sensitive than plain radiographs for early detection of an increase in skeletal vasculature, altered osteogenesis and abnormalities of the growth plate.[32,34–38] It also carries a lower total radiation dose than whole body skeletal survey. The incidence and relevance of asymptomatic 'hot spots' in a normal childhood population are unknown.

Wilson *et al.*[39] noted that plain radiographs are more sensitive for detecting cortical hyperostoses, and recommended annual lateral thoracic spine X-rays (less radiation than a bone scan) with supplementary films of symptomatic areas. Melnik[11] noted that the cervical region is most commonly affected in etretinate-induced spinal hyperostosis. Also, DiGiovanna pointed out that skeletal abnormalities are more commonly peripheral than spinal.[10] As radiographs are better at detecting minor skeletal changes, such as cortical hyperostoses and ligamentous calcification, a selective skeletal survey is a more suitable baseline investigation. Bone scanning is more appropriate for the detection of growth plate disturbances or ossification of the brachial interosseous membrane.

Etretinate therapy has been used for the treatment of severe ichthyoses in children at our hospital, since 1980. Approximately 90% of our patients with NBIE and LI had a good clinical response to etretinate with a marked reduction in scaling and a modest reduction in erythema and pruritus. The four patients with SLS showed extensive shedding of thickened scaly skin with a considerable improvement in their appearance. Although neurological disease was unaffected, the skin changes led to an improvement in limb mobility. Improvement in BIE was less dramatic, mainly because patients appear particularly susceptible to the increased skin fragility that etretinate can induce. All three patients with Netherton's syndrome suffered deterioration of their skin condition whilst on etretinate and treatment was stopped. Very low-dose therapy with retinoids may be more effective in this condition.[40]

Growth in all our patients on long-term etretinate has been normal. This has also been confirmed in other studies.[15,25,27] It is apparent that some severely affected children suffer from growth retardation as a result of their chronic disease, and growth may improve after starting etretinate therapy.[16,38]

None of our baseline studies influenced the decision to commence therapy. Also, none of the follow-up studies altered our decision to continue treatment, as those defects which were detected were not considered clinically relevant.

Our experience over the past 11 years has therefore been reassuring with regard to the safety of etretinate. In our opinion, it is important that the dose is restricted to less than 1 mg/kg/day and that pretreatment skeletal surveys (excluding skull views) are performed in all patients. In future, we will confine regular follow-up radiology to those with pretreatment radiological abnormalities, and to those who develop musculoskeletal symptoms during treatment. Routine follow-up studies will not be undertaken.

Etretinate has revolutionized the lives of many children with severe ichthyosis. Our findings have shown that long-term etretinate treatment is well tolerated in children and that skeletal toxicity need not be a significant problem.

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British Journal of Dermatology (1992) 127, 392–395.

Bath-water compared with oral delivery of 8-methoxypsoralen PUVA therapy for chronic plaque psoriasis

P.COLLINS AND S.ROGERS
City of Dublin Skin and Cancer Hospital, Hume Street, Dublin 2

Accepted for publication 15 May 1992

Summary Forty-four patients with chronic plaque psoriasis were randomly allocated to treatment with bath-water-delivered 8-methoxypsoralen (bath 8-MOP) or oral 8-methoxypsoralen (oral 8-MOP) PUVA therapy. There was a significant reduction in extent of the lesions and psoriasis area and severity index (PASI) after 20 treatments with each modality. There was a fourfold reduction in cumulative ultraviolet A (UVA) dose in the bath group. Side-effects of erythema and nausea were less with bath therapy.

Although in the rest of Europe,[1] and in America,[2] 8-MOP is administered orally as part of PUVA therapy, the Finns have favoured topical PUVA using 4,5,8-trimethylpsoralen (TMP).[3] They have shown oral 8-MOP and bath TMP to be equally effective.[4] We recently reported our experience with bath 8-MOP in an open study, and showed that it was effective, and popular with patients.[5] The present study was set up to compare the efficacy and side-effects of bath and oral 8-MOP PUVA therapy.

Patients and methods

The study was approved by the Hospital Ethics Committee. Patients with chronic plaque psoriasis, not previously treated with PUVA, were recruited from out-patient clinics between July 1990 and May 1991. Patients were over 18 years old, had greater than 10% extent of psoriasis, and were not pregnant or lactating. They were randomly allocated to either treatment group, using a table of random numbers. Emulsifying ointment was the only topical agent used for 2 weeks before the study, and throughout.

Clinical grading of psoriasis severity

Psoriasis was assessed for extent by the rule of nines,[6] and by the PASI score.[7] These assessments were performed by the same person (P.C.) throughout the study, single blind. Patients were asked at each visit about nausea and pruritis. Scaling, erythema and infiltration were graded on a standard 0–3 scale: 1, mild; 2, moderate; 3, severe. The end-point was 20 treatments. Clearing was defined as complete resolution or minimal residual psoriasis (<1% extent). Patients were withdrawn if they were intolerant of treatment. Treatment was discontinued at the point of clearing or at 20 treatments.

8-Methoxypsoralen

Twenty-seven millilitres of a 1·12% solution of 8-MOP (Deltasoralen, Delta Laboratories, Dublin) were added to 80 l of bath water at 37°C to give a concentration of 3·78 mg/l. Patients soaked in the bath for 15 min, patted the skin dry with a towel, and received UVA immediately. Those on oral therapy took 8-MOP tablets (methoxsalen, Promedica) 0·6 mg/kg, 2 h before exposure to UVA. Whole body UVA was given in a Waldmann 8001 cabinet which contained 27 UVA fluorescent tubes. The irradiance of the tubes was 8–9 mW/cm^2 UVA during the study period. Patients wore UVA protective goggles and males wore genital protection in the cabinet. They were treated three times/week. Only patients taking oral 8-MOP wore UVA protective spectacles on treatment days.

The initial dose of UVA was based on standard skin types. The dose was increased after every third treatment, if indicated, in a stepwise fashion depending on skin type and response to treatment (Table 1).

Statistical methods

PASI scores are not interval data and therefore medians are used in place of mean values. The paired *t*-test was used to assess reduction in extent (mean and 95%

Correspondence: Dr P.Collins, Photobiology Unit, Department of Dermatology, Ninewells Hospital and Medical School, Dundee, Scotland DD1 9SY, U.K.

Table 1. UVA dosimetry for bath and oral 8-MOP PUVA

	UVA dosimetry J/cm^2	
Skin type	Starting Dose	Increments
Bath		
I	0·2	0·1*
II	0·3	0·1*
III	0 5	0·5
Oral		
I	1·5	0·5
II	2·5	0·5
III	3·5	1·0

*Increments of 0·5 J/cm^2 were made once the dose reached 0·5 J/cm^2.

confidence interval) within each group, and the Wilcoxon rank test for PASI score (median and 95% confidence interval). The reduction in extent between the bath and oral groups was compared using the independent *t*-test. The reduction in PASI median scores was compared using the paired Mann–Whitney *U*-test. The cumulative doses with each modality could not be compared statistically because starting doses and increments were different in each group.

Results

Forty-four patients were treated, 22 in each group. There were 12 males and 10 females in the bath 8-MOP group, with a mean age of 30·2±9·6 years (range 18–49 years). Five were skin type I, twelve type II and five type III. The oral 8-MOP group comprised 13 males and nine females with a mean age of 36·3±14·2 years (range 18–69 years). Five were skin type I, 11 type II, and six type III. Overall, 74% of patients were skin types I and II. Four patients discontinued treatment, two from each group. One defaulted, two had PUVA pain (one in each group) and one flared on oral therapy. Of the remaining 20 in each group, the mean extent in the bath group before treatment was 24·7±12·8% compared with 25·2±16·7% in the oral group. The PASI scores were comparable, and values before and after treatment are illustrated in Figure 1 and listed in Table 2. The reduction in extent (mean and 95% confidence interval) in the bath group was 18·6 (11·4–25·9) and was 22·0 (14·4–29·5) in the oral group. There was no significant difference between the groups. The reduction in PASI scores (median and 95% confidence interval) in the bath group was 10·4 (6·85–12·55), and 11·0 (9·15–13·6) in the oral group, and the difference was not significant.

Fourteen patients in each group cleared (Table 3).

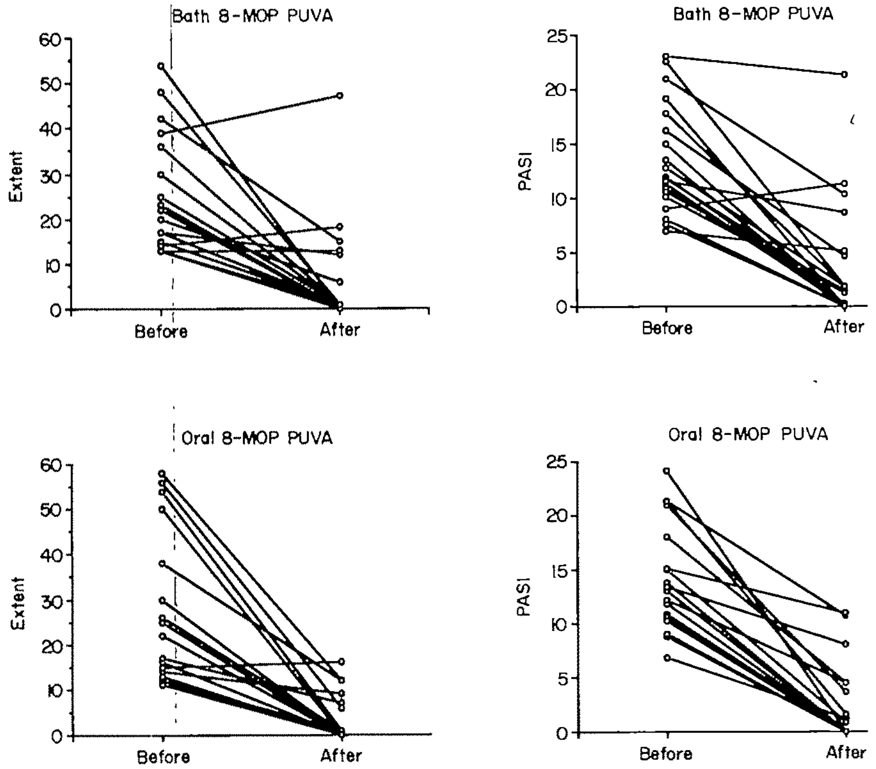

Figure 1. Extent and PASI scores before, and at the end of, treatment with bath and oral PUVA.

Table 2. Extent and PASI scores before and after treatment

	Extent		PASI	
Exposures	Before	After	Before	After
Bath 8-MOP PUVA				
10	13	0	7·6	0
17	25	1	11·9	0·3
9	25	1	11·0	0·3
18	36	1	17·8	1·8
20	15	1	10·6	1·5
14	30	1	11·8	1·2
9	22	0	11·4	0
17	23	0	15·0	0
16	54	0	19·2	0
20	17	1	10·1	1·2
20	13	1	12·8	1·8
17	13	0	8·0	0
18	13	0	13·5	0
20	48	1	22·6	1·8
20	17	12	11·6	8·6
20	13	13	6·9	5·1
20	14	18	9·0	11·4
20	20	6	16·2	4·6
20	39	47	23·0	21·4
20	42	15	21 0	10·4
Oral 8-MOP PUVA				
20	12	1	6·8	1·2
11	12	0	10·8	0
20	54	1	21·4	1·6
14	22	0	13·0	0
14	12	0	9·0	0
14	11	0	8·8	0
20	12	0	10·4	0
13	13	0	10·6	0
12	30	0	13·8	0
15	11	0	9·0	0
19	26	1	15·0	0·8
16	50	0	24·2	0
12	25	0	11·8	0
7	16	0	10·2	0
20	14	9	12·2	4·5
20	56	6	21·0	3·6
20	17	7	13 4	8·1
20	38	12	21·3	10·7
20	15	16	15·1	11·0
20	58	12	18·0	4·5

Table 3. Clearing data of patients on bath and oral PUVA therapy

Number of exposures (mean ± SD)	Cumulative dose J/cm² UVA (mean ± SD)
Bath ($n=14$)	
16·1±4·1	14·5±9·8
Oral ($n=14$)	
14·8±3·9	60·1±25·7

Each group required a similar number of exposures to clear and the cumulative dose was greater in the oral group by a factor of four.

Side-effects were more prevalent in the oral group. Seven (31·8%) had grade 2 erythema on oral, compared with three (13·6%) on bath therapy. Nine (41·9%) in each group had pruritus, which was mild, and only half required antihistamines. Nine patients (40·9%) on oral therapy had mild nausea.

Discussion

We have previously shown in an open study that bath 8-MOP PUVA is effective in clearing plaque psoriasis.[5] In this present study, bath 8-MOP and oral 8-MOP PUVA were equally effective, using mean reduction in extent to assess the response. The reduction in extent for both groups is similar to that reported by Lowe.[8] The mean cumulative UVA dose with oral therapy is four times higher than with bath treatment. A similar differential has been noted by other workers when comparing bath TMP,[4] or bath 8-MOP,[8] with oral 8-MOP PUVA. In these studies the mean cumulative UVA doses for both oral and bath treatment were two to three times higher than ours. However, they included all patients, whether they cleared or failed to clear, whereas we counted only those who cleared. Other reasons include different dosimetry and fewer patients with skin types I and II.

Stern has shown that as the number of exposures to PUVA increases and exceeds 260 there is an increased risk of skin cancer.[9] A recent Swedish study[10] suggests there is a real risk of skin cancer with more than 200 exposures and a cumulative dose of 1200 J/cm² UVA. Cumulative UVA dose in relation to skin type is likely to be a more accurate predictor of skin cancer risk than the number of treatments, as dosimetry is not standardized.[11] It is of interest that the Finns have found no skin cancer in 1000 patients treated with bath TMP over an 11-year period.[12] Skin cancer might be expected, as topical TMP is more phototoxic than oral 8-MOP PUVA.[4] It may be that TMP is less carcinogenic than 8-MOP,[13] or that the lower cumulative UVA dose with bath TMP lessens the risk of skin cancer. A similar reduction in cumulative UVA dose was achieved with bath 8-MOP in this study.

Topical PUVA has been associated with more local side-effects than oral 8-MOP PUVA, and it was therefore surprising that grade 2 erythema was less common in the bath (13·6%) compared with the oral (31·8%) group.[4,8] Thirty-five per cent of Lowe's bath 8-MOP group had grade 2 erythema, but he used more aggressive dosimetry.[8] Our rate of pruritus (40·9%) for both groups was higher than that usually reported (25%),[1] but as patients were asked about side-effects at every visit the results included even mild and transient cases.

One of the drawbacks with oral PUVA, from the patient's point of view, is the need to wear protective spectacles on treatment days.[5] Patients in the bath group did not wear them, because systemic absorption of psoralen is minimal with bath 8-MOP. This is supported by the findings of two recent studies.[14,15] Other advantages of bath PUVA are the absence of nausea and the reduced time spent in the PUVA cabinet. One disadvantage is that the cost of the drug per treatment is three times that of oral 8-MOP. Another is that to replace showers, which are standard in most day-care units, with baths, would be costly. Hence, although bath PUVA is effective it will probably be used mostly as an alternative to oral 8-MOP, unless it proves to be less carcinogenic in the long term.

Acknowledgment

We thank Dr Bernard McCartan and Dr Myra O'Regan for their help.

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British Journal of Dermatology (1992) 127, 396–400.

An investigation of the pharmacokinetics of topical terbinafine (Lamisil®) 1% cream

S.HILL, R.THOMAS, S.G.SMITH* AND A.Y.FINLAY

Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.
**Medical Department, Sandoz Products Limited, Frimley Business Park, Frimley, Camberley, Surrey GU16 5SG, U.K.*

Accepted for publication 12 April 1992

Summary

Twenty human volunteers were entered into a study to investigate the pharmacokinetics of terbinafine 1% cream. Subjects were randomized to receive terbinafine 1% cream applied to the back on 1 day, or on 3, 5 or 7 consecutive days. Up to five sequential skin surface biopsies were taken at a site on the upper back at various time-points both during treatment, and following cessation of treatment. Terbinafine levels in these biopsies were analysed using HPLC. The study showed that increasing the number of applications from one to seven did not significantly increase the peak concentration (C_{max}) in the stratum corneum. It did, however, increase the total amount of terbinafine found in the stratum corneum, resulting in terbinafine being detected for longer periods after cessation of therapy. Treatment for 7 days resulted in terbinafine still being detectable 7 days after cessation of therapy, when the terbinafine concentration was significantly higher than the known cidal concentrations for the common causative organisms of superficial dermatomycoses. This study indicates a significant potential for short-duration treatment with terbinafine (Lamisil®) 1% cream in superficial dermatomycoses.

Terbinafine (Lamisil®) is a synthetic antimycotic agent from a new class of compounds, the allylamines. *In vitro* studies have shown that terbinafine exerts a primary fungicidal action against dermatophytes and a number of yeasts and moulds.[1,2] Clinically, both oral and topical formulations of terbinafine have been shown to be highly effective against superficial dermatophyte infections.[3,4]

In a double-blind study comparing terbinafine 1% cream with clotrimazole (Canesten®) 1% cream in the treatment of tinea pedis over a 4-week period, it was observed that, in patients treated with terbinafine, fungal cultures became negative significantly sooner than in those treated with clotrimazole.[5]

More recently, placebo-controlled studies of 14-day treatment of tinea pedis[6] and 7-day treatment of cutaneous candidiasis, tinea corporis and tinea cruris[7] have shown terbinafine to be highly effective when used for this short duration. This information, combined with its known fungicidal activity, raises the possibility that even shorter durations of therapy with terbinafine may be possible in the treatment of these conditions.

To complement clinical studies investigating the minimum duration of therapy necessary for tinea infections, this present study was established to compare the pharmacokinetics of terbinafine 1% cream when applied to the back of healthy volunteers on 1 day, or on 3, 5 or 7 consecutive days. The aims of the study were to determine whether repeated applications increased the concentration of terbinafine in the stratum corneum, to determine how long and at what concentration terbinafine remained in the stratum corneum after cessation of treatment, and whether this was affected by the duration of treatment given.

Methods

Subjects

Ten male and 10 female subjects (median age 40 years, age range 22–62) were recruited to the study. Subjects to be included were healthy males or females (who were not pregnant or lactating, and if of a reproductive age, were using reliable contraceptive measures), aged 18 years or older, who had given written witnessed informed consent. Subjects were excluded if they had widespread skin disease, abnormal skin, a very hirsute upper back, had any systemic disease, were using any

Correspondence: Dr A.Y.Finlay, Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

topical application or had a history of reactions to other topically applied products.

Subjects were randomized into four groups of five individuals to receive a single application of terbinafine 1% cream on 1 day, or on 3, 5 or 7 consecutive days. Two areas measuring 12 × 8 cm were marked on the back of each subject, and 0·5 g of terbinafine 1% cream was applied over the two areas on 1 day, or on 3, 5 or 7 consecutive days. The cream was allowed to dry undisturbed for 30 min.

Skin surface biopsies

Skin surface biopsies (SSBs) with a surface area of between 6 and 10 cm^2 were taken using the technique described by Marks and Dawber.[8]

Prior to the first application a SSB was taken from all subjects. Subjects who were on the 3-day regimen had a SSB taken prior to the application on the third day. Subjects who were on the 5-day regimen had a SSB taken immediately prior to the applications on the third and fifth day, and subjects on the 7-day regimen had a SSB taken prior to applications on the third, fifth and seventh days.

All subjects had SSBs 4, 8, 12, 24, 48, 72, 96 and 168 h (7 days) following the last application.

A maximum of five sequential SSBs were taken at each site at each time-point. The uppermost layer is referred to as Level 1 and the lowermost layer as Level 5.

Sample analysis

SSBs were placed in 2 ml of methanol for a period of 2 h. The SSBs were then removed and washed with a further 2 ml of methanol to ensure complete extraction of terbinafine. The methanol washes were combined, evaporated and the residue redissolved in 0·2 ml of mobile phase. A volume of 0·1 ml was then injected into the HPLC. Levels of terbinafine were measured and integrated using the JCL 6000 Data Acquisition PC System (Jones Automatography). The level of detection of the assay was 0·18 ng/cm^2 and the level of quantification <0·48 ng/cm^2, with a precision of 5%. Analysis was carried out blind to treatment regimen, after the last biopsy was taken from the last subject.

Statistical methods

Total stratum corneum drug concentration was calculated by summing the individual stratum corneum levels for each time-point for each subject.

Calculation of areas under concentration (AUC) versus time curves. The AUC from 0 to 168 h ($AUC_{0\text{–}168\ h}$) was calculated for each level, and the total for each subject, using the trapezoidal rule.

Estimation of peak concentration (C_{max}). Graphs for stratum corneum drug concentrations versus time were prepared and the maximum drug concentration (C_{max}) identified visually, for each stratum corneum level, and for the total stratum corneum, for each subject.

Calculation of elimination half-life ($t\frac{1}{2}$). Stratum corneum concentrations for the descending part of the stratum corneum drug concentration versus time curves (i.e. from 4 to 168 h after the last drug application) were 'log-transformed' (natural logarithms) and log × drug concentrations versus time plots constructed. In nearly all cases two different exponentials were present. The first of these (elimination Phase I) comprised the data points at 4, 8 and 12 h. The remaining data points (i.e. 24 h and later) comprised elimination Phase II. Therefore, for consistency, linear regression models were fitted to the data points at 4, 8 and 12 h to obtain the elimination rate constant. Elimination half-lives ($t\frac{1}{2}$) were then calculated from the elimination rate constant using the following equation:

$$t\tfrac{1}{2} = \frac{\log 2}{\text{elimination rate constant}}.$$

Results

Following application to the skin, peak concentrations of terbinafine were attained after 4 h. This was true for the individual levels of the stratum corneum as well as the total concentration.

Mean total stratum corneum concentrations (C_{max}) are shown in Table 1. Mean stratum corneum C_{max} for Levels 1–5 are shown in Figure 1. The C_{max} decreases with descent through the stratum corneum from upper to lower levels. For stratum corneum Levels 1–3, and the total stratum corneum levels, there appears to be a treatment duration increase in C_{max}. However, for Level 4 no such duration-dependent trend is apparent and Level 5 appears to show a reversal of the trend, with the 1-day group having higher levels.

Mean total stratum corneum $AUC_{0\text{–}168\ h}$ concentrations are presented in Table 1. Mean $AUC_{0\text{–}168\ h}$ for Levels 1–5 are shown in Figure 2. These data show an apparent increase in this parameter with duration of treatment for the total, and for Levels 1–4. For Level 5 no such increasing trend is apparent.

Table 1. Summary of pharmacokinetic parameters for total stratum corneum concentrations

Treatment group	C_{max} (ng/cm^2)	AUC$_{0-t}$ (ng/cm^2/h)
1-day		
Mean	949	9694
SD	105	1234
3-day		
Mean	966	10,321
SD	52	948
5-day		
Mean	1049	12,544
SD	57	816
7-day		
Mean	1094	13,492
SD	26	695

A two-dimensional kinetic model was found to be the best fit to the individual patient data. From this, elimination half-lives were calculated and are presented in Figure 3.

The mean total stratum corneum terbinafine concentrations in ng/cm^2 are shown in Figure 4 (a) and (b). These data indicate that extending the treatment duration increases the time taken to fully eliminate the drug from the stratum corneum.

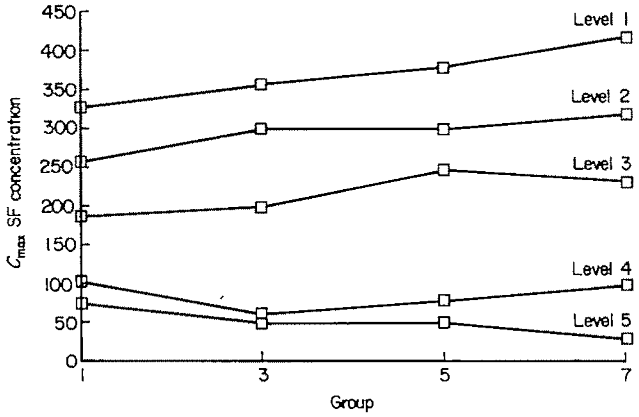

Figure 1. Maximum terbinafine concentration (C_{max}) in ng/cm^2 by level over the four treatment groups.

Discussion

The first objective of the study was to determine whether repeated applications of terbinafine (Lamisil®) 1% cream increased the concentration of terbinafine in the stratum corneum. The results for the maximum concentration (C_{max}) in the stratum corneum indicate that increasing the number of applications from one to seven increases the maximum concentration in the stratum corneum. However, the increase in mean concentration with seven applications compared with one application was only 15%. Looking at the maximum concentrations found in the individual levels of the stratum corneum (Fig. 1), the Level 1 results mirror those of the total. However, descending through the levels of the stratum corneum, the duration/concentration relationship becomes less clear, until at the lowest level (Level 5) there is a reverse trend, the highest concentrations being found in the 1-day group. This could partly be explained by the practical difficulties in obtaining consistent samples at this lowest level. With longer periods of treatment, the stratum corneum becomes more

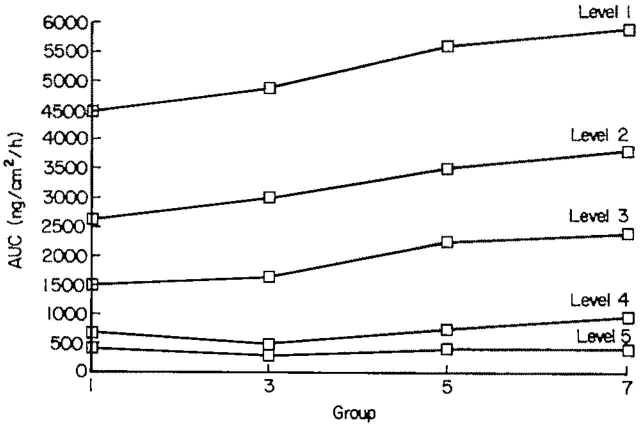

Figure 2. AUC in ng/cm^2/h by level over the four treatment groups.

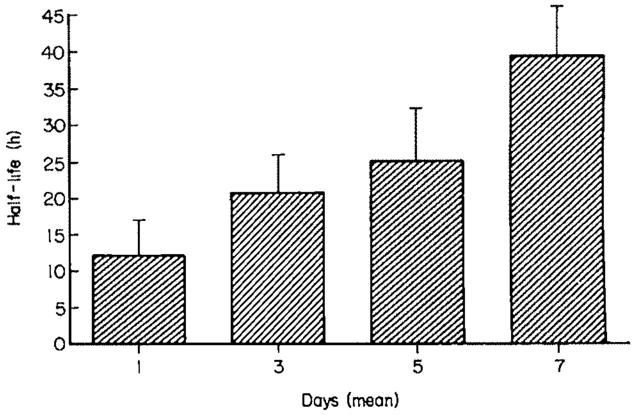

Figure 3. Summary of half-lives (h). ▨, mean; bars, SD.

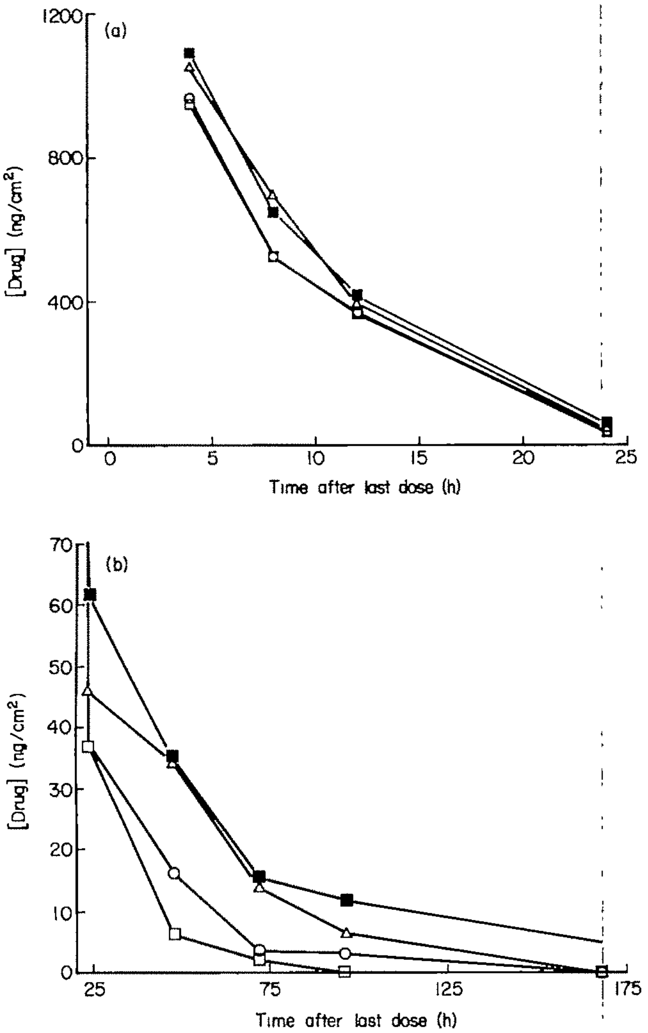

Figure 4. (a) Mean total drug concentrations in stratum corneum for each group (elimination Phase I). (b) Mean total drug concentrations in stratum corneum for each group (elimination phase II). —□—, G1; —○—, G3; —△— G5; —■—, G7.

saturated, and this may result in more stratum corneum being removed by SSB at each level in those volunteers treated for longer periods. Therefore, at Level 5 there may have been less consistency in the quality of the samples, particularly in volunteers treated for longer periods, and this may have contributed to the variability of these results.

There was a clear duration response both in the total concentrations and in Levels 1, 2 and 3 (Fig. 2). Levels 4 and 5 showed very little duration response. The total stratum corneum AUC_{0-t} concentration for seven applications was approximately 40% higher than for one application.

The second objective was to determine for what duration, and at what concentrations, terbinafine remained in the stratum corneum following cessation of treatment, and whether this was affected by the duration of treatment. There is an apparent increase in the elimination half-life as the number of applications is increased (Fig. 3). There was a threefold prolongation of the half-life after 7-day treatment compared with the 1-day treatment. This conclusion is supported by the data for total elimination. Following one application, terbinafine is not detectable 96 h (4 days) after stopping treatment (Fig. 4b). Following 3 and 5 days' treatment, terbinafine is not detectable between 96 and 168 h (7 days) after treatment. However, following 7 days' treatment, detectable levels are still found 168 h (7 days) after cessation of treatment.

Further points have been raised by the data which were not part of the original objectives, but warrant further discussion. The elimination is biphasic with a rapid elimination over the first 12 h and then a gradual elimination over the following 1–7 days. This has been seen with other topically applied compounds.[9] This is likely to be because initially, following application, the stratum corneum is over-saturated with terbinafine. This over-saturated concentration is then lost very rapidly (elimination Phase I) leaving a smaller amount that is bound to, or held in, lipid components in the stratum corneum. This amount is then lost more slowly (elimination Phase II). During the elimination phase, the terbinafine was lost initially from the lower levels. This is probably via two routes:

(a) diffusion from the lower levels of the stratum corneum to the upper levels along a concentration gradient;

(b) replacement of the lower cell layers of the stratum corneum from the germinal layer and the erosion of the upper layers of the stratum corneum.

It is also important to ascertain the clinical relevance of these data. It is known that the fungicidal concentrations of terbinafine required to kill common skin dermatophyte organisms such as *T. rubrum* are approximately 0·003 μg/ml.[1] We can compare the levels found in the stratum corneum in this study with the fungicidal concentrations using the following assumptions:

Estimated thickness of 1 cm^2 surface area of
skin surface biopsy = 2·5 μm (0·0025 mm),
therefore volume of SSB = 10 × 10 × 0·0025 mm
= 0·25 mm^3,
therefore 1 ng/cm^2 SSB = 1 ng in 0·25 mm^3 or 4 ng in mm^3,
as 1000 mm^3 = 1 ml,
then 1 ng/cm^2 SSB = 4 μg/ml.

Therefore, a concentration of 1 ng/cm^2 is approximately

1000 times higher than the known fungicidal concentration for *T. rubrum* and concentrations of 0·33 ng/cm^2 detected 7 days after cessation of therapy are still 100 times the cidal concentration. It should also be noted that the detection level of the assay is approximately 0·2 ng/cm^2 (or 0·8 μg/ml) and thus this does not preclude fungicidal concentrations of terbinafine remaining in the stratum corneum for longer than was shown in this study.

In conclusion, increasing the number of applications did not significantly increase the maximum concentration of terbinafine found in the stratum corneum. It did, however, increase significantly the total concentration, which resulted in detectable levels of terbinafine being found in the stratum corneum for longer periods after cessation of therapy. The levels detected after cessation of treatment were significantly higher than the known cidal concentrations for terbinafine against dermatophytes. This indicates a significant potential for short treatment duration, or the use of lower concentrations of topical terbinafine (Lamisil®) in the management of superficial dermatomycoses.

Acknowledgment

The authors would like to thank Mr R.Budhram of BIOS (Consultancy & Contract Research) Limited, Pinewood, College Ride, Bagshot, Surrey, for carrying out the pharmacokinetic analysis.

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British Journal of Dermatology (1992) 127, 401–402.

Beta-thalassaemia minor and porphyria cutanea tarda

K.E.McKENNA AND J.F.DAWSON*

Department of Dermatology, Belfast City Hospital, Belfast
**Department of Dermatology, Ulster Hospital, Dundonald, Belfast, Northern Ireland*

Accepted for publication 15 May 1992

Summary

A case of porphyria cutanea tarda occurring in association with beta-thalassaemia minor is reported in a 33-year-old Northern-Irish woman. Aetiological factors in this case included oral and parenteral iron therapy for refractory anaemia which was subsequently diagnosed as beta-thalassaemia minor, and the use of an oestrogen-containing oral contraceptive pill.

Porphyria cutanea tarda (PCT) results from partial inactivation of uroporphyrinogen decarboxylase, an enzyme involved in haem biosynthesis in the liver.[1] Porphyrins accumulate in the liver, pass into the blood and are excreted in stool and urine. Photosensitization of the skin occurs due to tissue accumulation of porphyrins. Clinical features include skin fragility, bullae, pigmentation and hypertrichosis.[2] Although it is usually a sporadic disease, autosomal dominant inheritance is recognized. Iron is well recognized in the pathogenesis of PCT, and hepatic siderosis is common.[2,3] Other factors in pathogenesis include alcoholic and non-alcoholic liver disease, oestrogen use, including the combined oral contraceptive pill[4] and, rarely, exposure to toxic hydrocarbons. We present a case of PCT in a female patient with a long history of microcytic anaemia which was subsequently diagnosed as beta-thalassaemia minor. In addition she had been taking an oestrogen-containing oral contraceptive pill.

Case report

A 33-year-old woman presented in October 1989 complaining of painful blistering of the backs of her hands for the previous 6 months. There was no prior history of skin disease. She had a long history of anaemia which had worsened during each of her three pregnancies. She had been treated with oral iron on frequent occasions. During her first pregnancy in 1983 she was also treated with intravenous iron, and required a blood transfusion post-partum. There was no known family history of anaemia or skin disease. Her current drug therapy included Fefol® one capsule daily (ferrous sulphate 150 mg, folic acid 500 μg) and the combined oral contraceptive pill, which she had been taking since her early twenties. Her alcohol consumption was not excessive and she was not in contact with toxic substances.

On examination there were small atrophic scars on the dorsa of her hands, with several small blisters and a few milia. There was no hyperpigmentation, hypertrichosis or hepatosplenomegaly.

Investigations revealed a haemoglobin of 11 g/dl; mean corpuscular volume (MCV) 69 fl (normal 84–99); mean cell haemoglobin (MCH) 21·6 pg (normal 27–32); mean corpuscular haemoglobin concentration (MCHC) 31·2 g/dl (normal 30–35); white cell and platelet counts normal; reticulocytes 4% (normal 0–2); haemoglobin A2 5·3% (normal 1·5–3); haemoglobin F 2·2% (normal 0–2). These results were consistent with a diagnosis of beta-thalassaemia minor. Liver function tests showed a transiently raised alanine aminotransferase (ALT) at 57 IU/l (normal 8–35). Hepatitis B surface antigen was negative. A liver biopsy was not performed. Serum ferritin was 181·8 μg/l (normal 11–120). An excess of urinary porphyrins was detected: total porphyrins 2974 nmol/l (upper limit of normal 320); uroporphyrins 1253 nmol/l (normal <24); urinary coproporphyrins were normal. Faeces were positive for isocoproporphyrins. A diagnosis of PCT was made on the basis of these results.

The oral iron and contraceptive pill were both discontinued, and she was strongly advised to avoid alcohol. A high factor sunscreen was prescribed. The conventional method of treating PCT is by venesection,[5] but in view of her beta-thalassaemia venesection was not performed. Clinical remission of her PCT occurred within a period of 6 months. During this time the patient also developed troublesome menorrhagia. This caused depletion of her body iron stores and her serum ferritin returned to normal. This almost certainly accelerated her clinical

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Correspondence: Dr K.E.McKenna, Department of Dermatology, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland.

remission. Despite the onset of menorrhagia her haemoglobin level has been well maintained and serum ferritin has remained within normal limits.

On screening her three children all were found to have a microcytic blood picture and to have elevated levels of haemoglobin F. The children had no clinical evidence of PCT, and urinary porphyrins were not elevated.

Discussion

The association of PCT and beta-thalassaemia is an unusual combination and has rarely been reported.[6-10] The former is a disorder of haem synthesis and the latter of beta-globin chain synthesis.[1,11] Beta-thalassaemia can predispose to the development of PCT due to iron overload.[12,13] This can result from haemolysis, ineffective erythropoiesis,[14] increased absorption of iron from food[15] and iatrogenic administration of iron. Possible iron-induced hepatic toxicity in thalassaemia minor has been reported.[16,17] In addition, thalassaemia minor can result in increased susceptibility to hepatitis B virus infection and thus to chronic liver disease.[18]

In the case we have reported PCT was probably induced by a combination of chronic iron overload and the concomitant use of an oestrogen-containing oral contraceptive pill. Hepatitis B serology was negative. Amerio *et al.*[6] reported a case of a 57-year-old patient who also had diabetes mellitus, hypersideraemia and hepatic siderosis. Chapman[7] described a mother and daughter both of whom had beta-thalassaemia minor. The mother had PCT and the daughter had subclinical PCT. Both had hypersideraemia and hepatic siderosis. Ivanov *et al.*[8] reported a female aged 40 years with beta-thalassaemia who had been treated with iron preparations and subsequently developed PCT. Gibertini *et al.*[9] reported five cases of PCT occurring in carriers of the beta-thalassaemia trait. All were male and were aged between 29 and 57 years. Liver cirrhosis was diagnosed in three patients and chronic active hepatitis in two patients. Mild to severe hepatic siderosis was present in all cases. In four of the patients hepatitis B surface antigen was present. Alcohol abuse was present in only one patient. Gambari *et al.*[10] reported a 61-year-old woman with both disorders. There were no other aetiological factors. She had mildly disturbed liver function tests and hepatic siderosis.

It is interesting that in our patient clinical and biochemical remission was achieved by discontinuation of iron supplements and the oral contraceptive pill, combined with the onset of menorrhagia which caused depletion of body iron stores. If relapse of PCT should occur desferrioxamine chelation therapy would be appropriate. This treatment has been used successfully in the management of PCT occurring with beta-thalassaemia minor.[8,9]

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British Journal of Dermatology (1992) **127**, 403–406.

Phenytoin-induced pseudolymphoma. A report of a case and review of the literature

D.W.S.HARRIS, L.OSTLERE, C.BUCKLEY, S.WHITTAKER,* P.SWENY† AND M.H.A.RUSTIN

Departments of Dermatology and †Nephrology, The Royal Free Hospital and School of Medicine, London NW3 2QG, U.K.
**St John's Dermatology Centre, St Thomas's Hospital, London SE1 7EH, U.K.*

Accepted for publication 1 May 1992

Summary We report a patient with phenytoin-induced pseudolymphoma mimicking cutaneous T-cell lymphoma (CTCL). Despite withdrawal of phenytoin, there was persistence of the cutaneous eruption and lymphadenopathy. Southern blot analysis of immunoglobulin and T-cell receptor genes was therefore used to assess whether there was a clonal lymphoid expansion. However, no rearrangement of the beta T-cell receptor gene or immunoglobulin heavy-chain gene was detected in tissue DNA from skin and lymph nodes. One year later the patient became asymptomatic, although he is still at risk of developing a true malignant lymphoma in the future, a condition known as pseudo-pseudolymphoma. It is suggested that genotypic studies may help in the initial diagnosis and the subsequent management of such patients.

Phenytoin-induced pseudolymphoma is a rare condition comprising cutaneous and systemic features which resolve within 2–6 weeks of drug withdrawal. The majority of reported cases have occurred in Black patients. The dermatological manifestations include morbilliform, pustular, vesiculo-papular and follicular rashes which may affect the face, trunk and limbs, especially acrally. Rarely, the eruption can present as an erythroderma or toxic epidermal necrolysis. The systemic features comprise a triad of fever, lymphadenopathy and hepatitis.

Case report

A 25-year-old Black man presented in August 1990 with a 1-week history of fever and an itchy eruption, associated with marked facial oedema. On examination he was pyrexial (temperature 40°C) and had generalized erythroderma with pin-point pustulation over the face and trunk. There was marked facial swelling. He had generalized firm, grape-sized lymphadenopathy and 3 cm splenomegaly. Over the next 5 days desquamation occurred, but the rash persisted. He had a complex past medical history following the discovery of posterior urethral valves and hydroureter at the age of 2 years. This abnormality had resulted in chronic renal failure for which he had undergone two unsuccessful cadaveric renal transplants. It was following a native left nephrectomy and subsequent removal of a renal angle haematoma that the patient was treated with phenytoin 100 mg b.d. because he suffered a grand-mal fit post-operatively. The rash appeared 5 weeks later.

Abnormal investigations at the time of presentation included: haemoglobin 7·8 g/dl, white cell count $13{\cdot}8\times10^9$/l, with an eosinophilia of 46%, aspartate transaminase 66 U/l (5–40 U/l), alanine transaminase 240 U/l (35–130 U/l), urea 28·7 mmol/l, creatinine 1068 μmol/l, potassium 5·5 mmol/l. Culture of a swab from a pustule produced a scanty growth of *Staphylococcus aureus*. The following tests were normal or negative: throat swabs, monospot, ASO titre, blood cultures and serology for HTLV-1 and HIV-1.

A skin biopsy from the chest showed a diffuse lymphohistiocytic infiltrate in the superficial and papillary dermis in a perivascular and periadnexal distribution. Epidermotropism, with absence of Pautrier's microabscesses, was noted (Fig. 1). There were occasional multilobular and hyperchromatic lymphocytes seen within the dermis. A lymph node biopsy showed destruction of lymph node architecture (Fig. 2) and an infiltrate which contained many cells with cerebriform nuclei and mitotic figures. Immunohistochemical staining with UCHL-1 confirmed that the majority of the infiltrate was composed of T lymphocytes.

Correspondence: Dr D.W.S.Harris.

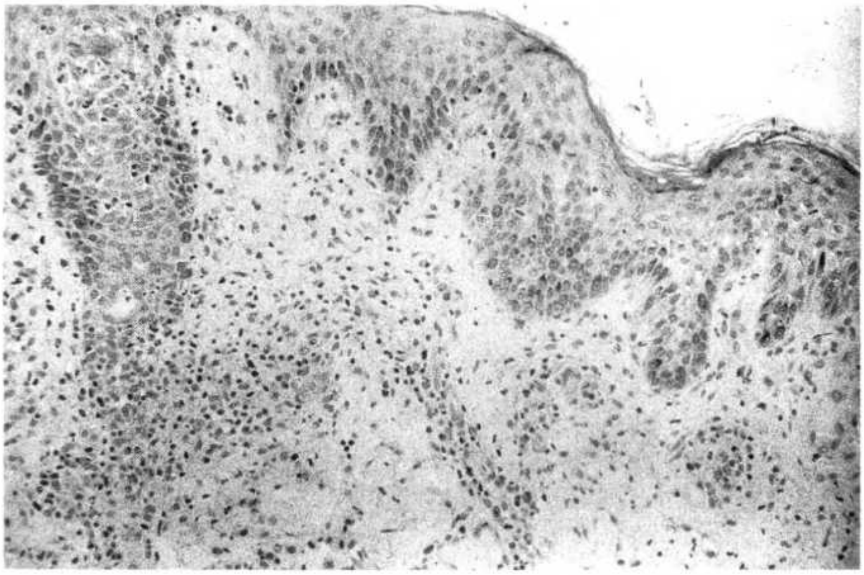

Figure 1. Skin biopsy showing a diffuse dermal lymphohistiocytic infiltrate with a periadnexal and perivascular distribution. Epidermotropism is seen but there are no Pautrier's microabscesses.

Figure 2. Low-power view showing destruction of the normal lymph node architecture and its replacement by the lymphohistiocytic infiltrate.

Based upon the histological features a diagnosis of cutaneous T-cell lymphoma was made. However, it was felt that the clinical picture was more suggestive of a phenytoin-induced pseudolymphoma. Tissue DNA was extracted from skin and lymph node biopsies using conventional methods.[1] DNA was digested with restriction enzymes EcoR1 and HInd 111, subjected to electrophoresis and Southern blot hybridization with probes for the constant region of the beta T-cell receptor gene and for the immunoglobulin heavy-chain gene under conditions of high stringency.[2] These studies consistently revealed a germline configuration and no discrete rearrangements of either the beta T-cell receptor or the immunoglobulin heavy-chain gene in tissue DNA from skin and lymph node biopsies.

The rash progressed, and new areas of pustulation appeared on the abdomen. Phenytoin was stopped and prednisolone 30 mg daily commenced. The erythroderma improved over the next 2 weeks but the pruritus persisted. A reduction in steroid dose to 15 mg daily provoked a severe flare of the rash. The patient was eventually weaned off steroids 11 months after presentation. During this period he was readmitted twice with septicaemia and signs of pustulation on his skin, from which a hospital-acquired multiresistant *Staphylococcus aureus* was cultured.

Discussion

Phenytoin-induced pseudolymphoma is an uncommon syndrome with fewer than 200 cases reported, despite world-wide use of the drug as an anticonvulsant.

The first report of hydantoin-induced lymphadenopathy by Coope and Burrows[3] appeared in 1940, 2 years after phenytoin was introduced. The adenopathy and accompanying fever disappeared on cessation of therapy, but recurred upon rechallenge. In 1959 Saltzstein and Ackerman[4] reviewed 75 cases and reported a further seven patients, two of whom subsequently died less than 5 years later of lymphoma. In 1966 Hyman and Somers[5] reported six patients on anticonvulsant therapy who developed a histologically proven Hodgkin's lymphoma or lymphosarcoma. All six patients were taking phenytoin in addition to other anticonvulsants. Withdrawal of phenytoin did not halt progression of the lymphoma. Gams *et al.*[6] later reported a case in which phenytoin-induced lymphadenopathy regressed after cessation of therapy. However, a fatal lymphoma developed 20 months later. This clinical course is known as pseudo-pseudolymphoma.

Four major types of phenytoin-induced lymphadenopathy are recognized: benign lymphoid hyperplasia, pseudolymphoma, pseudo-pseudolymphoma and malignant lymphoma. Distinction between these is made by consideration of the clinical course of the illness and histological examination of lymphoid tissue.

The clinical picture of the pseudolymphoma syndrome develops between 5 days and 3 months (with an average of about 3 weeks) after taking phenytoin. The onset of this hypersensitivity reaction is heralded by a high spiking fever and and intensely pruritic erythematous maculopapular rash, occasionally progressing to an exfoliative erythroderma. Pustulation, occasionally generalized,[7] is a frequent finding, as is oedema, particularly of the face. The eruption resolves with desquamation. Lymphadenopathy can be marked, often with grape-sized nodes, and may be localized or generalized. There is evidence of hepatitis, with or without hepatomegaly. Splenomegaly is an occasional finding. Conjunctivitis, pharyngitis and a strawberry tongue may also be present. The peripheral blood shows a leucocytosis with eosinophilia. Haemolytic anaemia is a rarer finding. There is associated myalgia and arthralgia. These clinical features usually resolve over the same time period after withdrawal of the drug, but persistence of the symptoms is recorded, as occurred in our patient. Repeat administration of the drug results in recurrence of the syndrome. Patients who are at high risk of developing such reactions include those known to react to phenytoin who are re-exposed to the drug,[8] those who develop unrecognized signs whilst on the drug and who continue to take it,[9] and Black patients.[10]

Lymphoid hyperplasia and pseudolymphoma are clinically indistinguishable, but histological examination reveals normal lymph node architecture in the hyperplasia group. In the pseudolymphoma group, first described by van Wyk and Hoffman,[11] loss of normal architecture, focal necrosis and infiltration with eosinophils is seen. As in our case this histology may suggest an erroneous diagnosis of malignant lymphoma.

The pseudo-pseudolymphoma category applies to patients whose lymph node histology shows hyperplasia or malignant changes which regress upon stopping the drug. However, following an asymptomatic period they subsequently develop a malignant lymphoma. The lymphoma group have histological changes of a malignancy from the outset, and lack any sign of clinical regression. It appears unlikely that phenytoin is overtly carcinogenic in view of the large number of patients treated with this drug and the rarity of lymphomatous change. However, Li *et al.*[12] and Anthony[13] have shown an increased incidence of lymphoma occurring in patients on long-term phenytoin.

The analysis of immunoglobulin and T-cell receptor genes represents a definitive method for establishing the lineage and clonality of lymphoproliferative disorders, including cutaneous T-cell lymphomas. This technique has also proved valuable in the assessment of dermatopathic lymphadenopathy.[14] In order to clarify whether this patient had an underlying lymphoma, gene rearrangement studies were performed on lymph node and skin biopsies. This is the first reported use of such a technique in this difficult diagnostic situation. Analysis of DNA from both skin and lymph node showed no evidence of rearrangement of the T-cell receptor beta or immunoglobulin heavy-chain genes. This indicates that there was no evidence of a monoclonal lymphoid population, although the sensitivity of Southern blot analysis is such that a small lymphoid clone comprising 1% or less of the total cellular infiltrate could escape detection.[2] However, we suggest that this technique is the method of choice for assessing such patients and alerting the physician to the early transformation to lymphoma.

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British Journal of Dermatology (1992) 127, 407–410.

Centrifugal lipodystrophy of the face mimicking progressive lipodystrophy

Y.HAGARI, R.SASAOKA, S.NISHIURA, M.ISHIHARA, M.MIHARA AND S.SHIMAO
Department of Dermatology, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683, Japan.

Accepted for publication 9 April 1992

Summary

We report a Japanese boy with centrifugal lipodystrophy. The initial lesion developed on the forehead as an area of erythema which showed centrifugal spread and central fading to leave a residual depression. Three years later, similar erythematous lesions appeared on the cheeks, and he developed a cadaverous appearance. A central depression on the upper trunk was surrounded by an erythematous border and there was right cervical lymphadenopathy. Histological examination revealed inflammatory changes in the subcutaneous fat. Although this patient showed the characteristics of both centrifugal and progressive lipodystrophy (partial lipodystrophy), we consider that he has centrifugal lipodystrophy of the face because of the overall features of the lesions.

Centrifugal lipodystrophy is a rare disorder that was first described by Imamura *et al.*[1] in 1971, and was initially named lipodystrophia centrifugalis abdominalis infantilis. Thereafter, the designation of centrifugal lipodystrophy was proposed by Zachary *et al.*,[2] because the condition was also found to affect sites other than the abdomen, and to occur after infancy. A similar disorder involving fatty tissue has been designated as progressive lipodystrophy (partial lipodystrophy).[3,4] We report a case of centrifugal lipodystrophy who also had some features resembling progressive lipodystrophy.

Case report

In 1988, a 20-month-old boy was brought to our department with a 16-month history of dark-red, asymptomatic, erythematous lesions on the face. He was the product of a normal pregnancy, and delivery had been uneventful. A small area of erythema had first appeared on his forehead when he was 4 months old. It had enlarged to a violet-red macule, and its centre had subsequently faded, producing an annular configuration. Finally, the erythematous lesion completely disappeared, leaving a residual depression. When he was 14 months old, erythema developed on his right cheek after he was scratched by his sister. This lesion gradually enlarged and other areas of erythema subsequently developed on the left cheek.

Physical examination revealed a violet-red, infiltrative, oval erythematous lesion measuring 6 × 4 cm on the right cheek (Fig. 1a). There were multiple, irregular, poorly demarcated erythematous lesions up to 1 cm in diameter on the left cheek, and a slightly depigmented depression (5 × 3·5 cm) on the right side of the forehead (Fig. 1b). He was otherwise healthy.

Histology of a biopsy from the depression on the forehead demonstrated an almost normal epidermis, and a sparse perivascular inflammatory cell infiltrate in the dermis. There was loss of subcutaneous fatty tissue with a sparse inflammatory infiltrate (Fig. 2a). Large parts of the subcutaneous fat were replaced by fine, faintly eosinophilic bundles of collagen. The infiltrate in the subcutaneous fat was composed of lymphocytes, histiocytes, and a few giant cells (Fig. 2b). Biopsy of an erythematous lesion on the cheek showed a dense cellular infiltrate occupying the entire dermis and extending into the subcutaneous fat (Fig. 2c). The results of laboratory investigations including full blood count and renal and liver function tests were within normal limits. He was followed up, without any definite diagnosis being made.

Three years later, he was brought to our department again. On physical examination, he was 110 cm in height and 18·4 kg in weight, and presented a cadaverous appearance because of the loss of facial fatty tissue (Fig. 3a). Large, faintly violet-red, serpentine, erythematous lesions with a few fine scales and slight infiltration extended from his cheeks to the clavicular region across the neck, suggesting downward extension of the original

Correspondence: Dr Y.Hagari, Department of Dermatology, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683, Japan.

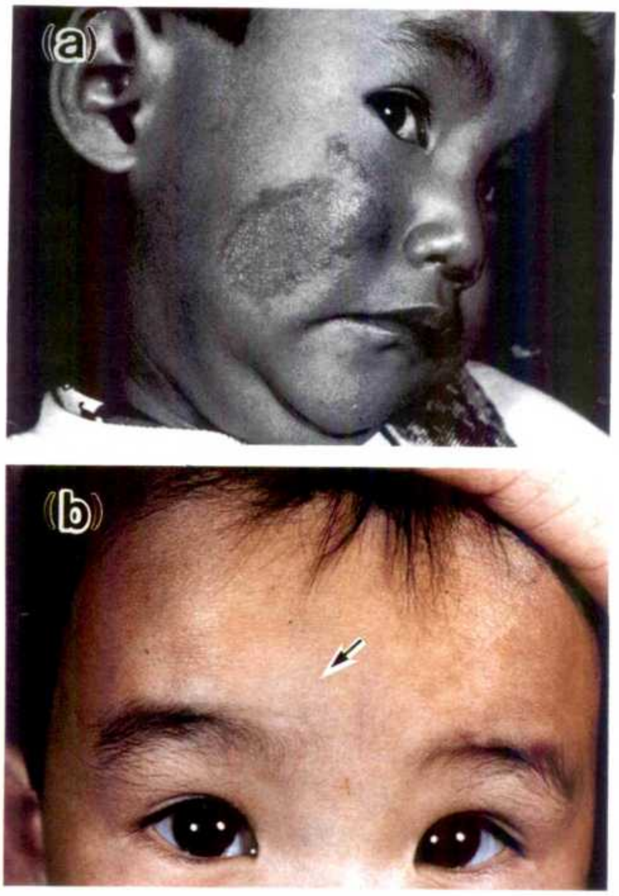

Figure 1. Clinical appearance at the first visit. (a) A violet-red, oval erythematous lesion with marked infiltration is seen on the right cheek. (b) A slightly depigmented depression is present on the right side of the forehead (arrow).

skin changes. The inner portions of the lesions were light-brown and slightly depressed, and the underlying veins were visible (Fig. 3b). Right cervical lymphadenopathy was also noted. Biopsy of the erythematous border demonstrated an almost normal epidermis, a sparse infiltrate in the dermis, and a moderately dense infiltrate composed of lymphocytes, histiocytes, foamy cells and a few plasma cells in the subcutaneous fat (Fig. 4a). Numerous microcysts composed of elongated histiocytes or foamy cells were observed at some sites in the fatty tissues (Fig. 4b). Laboratory investigations (full blood count, liver function, renal function, and complement components) again revealed no abnormalities. Serological testing for *Borrelia burgdorferi* was negative.

Discussion

Centrifugal lipodystrophy is a disorder affecting the trunk in childhood. The lesions show centrifugal extension with a slightly erythematous border and leave a depressed centre after resolution: there is associated regional lymphadenopathy.[1] On the other hand, pro-

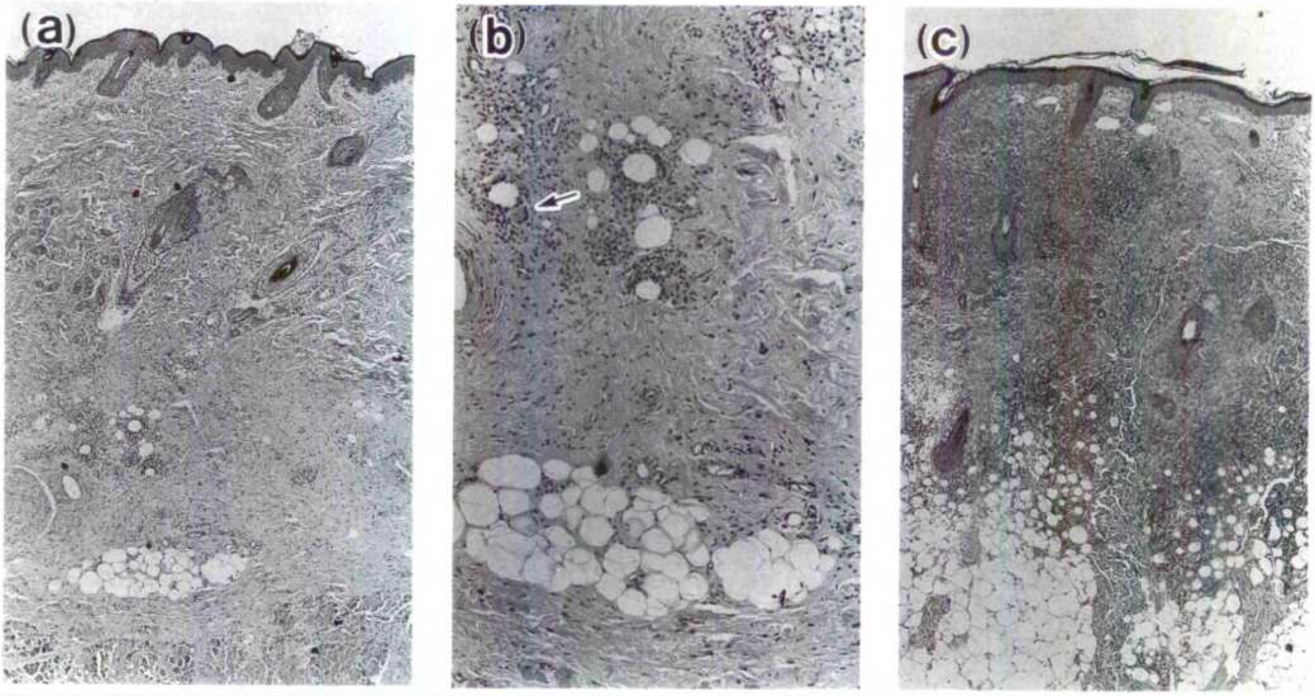

Figure 2. (a) Photomicrograph of a biopsy taken from the depressed lesion on the forehead. There is little or no infiltrate in the dermis. The amount of fatty tissue is apparently decreased (× 30). (b) High-power view of (a). Large portions of the subcutaneous fat are replaced by faintly eosinophilic fine bundles of collagen. The infiltrate in the fat is composed of lymphocytes, histiocytes, and a giant cell (arrow) (× 160). (c) Photomicrograph of the biopsy from the erythematous lesion on the cheek. A dense infiltrate is seen throughout the entire dermis, and extends into the subcutaneous fat (× 30).

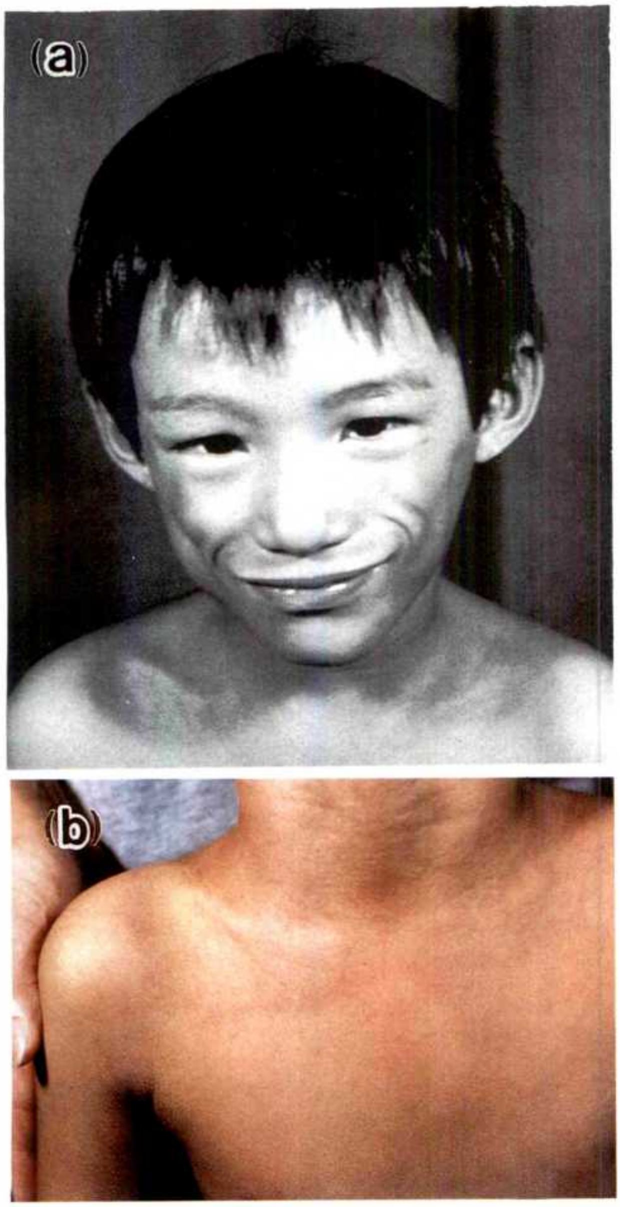

Figure 3. Clinical appearance at the second visit. (a) A cadaverous facies is now seen. (b) A faintly violet-red, gyrate erythematous area with a few fine scales is seen on the chest.

gressive lipodystrophy (partial lipodystrophy) is characterized by the symmetrical loss of fatty tissue on the face and the upper half of the body, producing a cadaverous appearance.[4]

The features of the two diseases and those of our patient are listed in Table 1. Both diseases have previously been clearly distinguished on the basis of the features shown in the Table. However, our patient possessed features characteristic of both centrifugal and progressive lipodystrophy. There are therefore two possibilities—our patient may have either of the two disorders in an atypical form, or alternatively, he may have another disease entity that is an intermediate between centrifugal and progressive lipodystrophy. The most notable features of our patient's lesions were a central depression surrounded by an erythematous border, centrifugal progression and inflammatory changes in the fatty tissues. These characteristics correspond to those of centrifugal lipodystrophy, and we therefore consider that he has this disease. Features such as the cadaverous appearance and the downward extension of the disease are considered to have developed because the initial lesions occurred on the cheeks.

The histological appearances of the forehead lesion at the first visit and the erythematous border at the second visit were also compatible with a diagnosis of centrifugal lipodystrophy.[5] However, it is difficult to explain the severe dermal and subcutaneous inflammatory cell

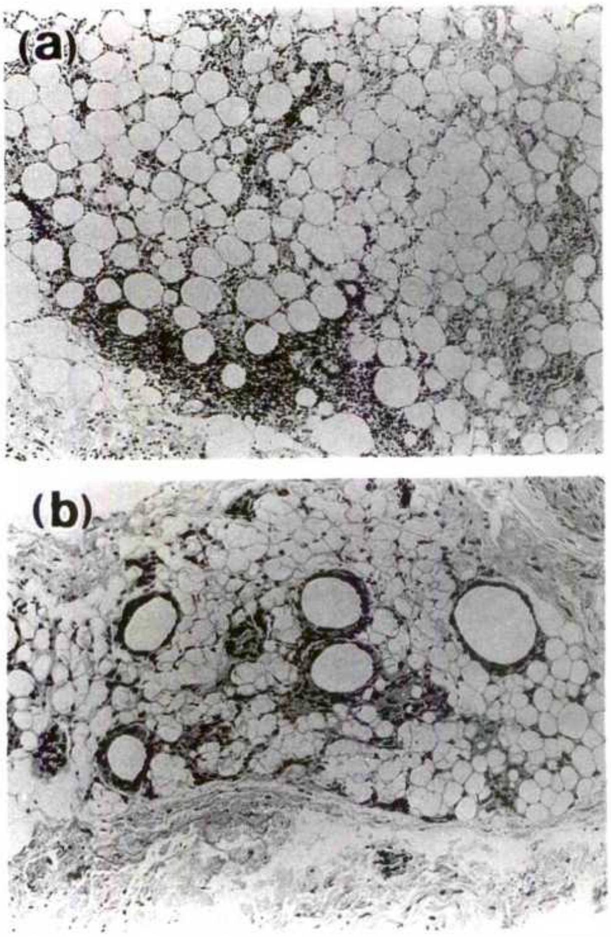

Figure 4. Photomicrographs from the biopsy of the erythematous border. (a) There is a moderately dense infiltrate composed of lymphocytes, histiocytes, foamy cells, and a few plasma cells in the fatty tissue (× 120). (b) The elongated histiocytes or foamy cells form numerous microcysts (× 80).

Table 1. Clinical and histological features of centrifugal lipodystrophy and progressive lipodystrophy

Features	Centrifugal lipodystrophy	Progressive lipodystrophy	Our patient
Initial site affected	Trunk	Face	Face
Preceding lesions	+	−	+
Cadaverous appearance	−	+	+
Symmetrical involvement	−	+	+ (nearly)
Mode of extension	Centrifugal	Downward	Centrifugal and downward
Erythematous border	+	−	+
Lymphadenopathy	+	−	+
Systemic involvement	− (usually)	+	−
Histological inflammation of fatty tissues	+	−	+

infiltrate in the cheek lesion noted at the first visit. To our knowledge, there is no precise histological data on the initial lesions of centrifugal lipodystrophy, even in a detailed review article by Imamura *et al.*[5] It is possible that this inflammatory infiltrate represented an initial lesion of centrifugal lipodystrophy modified by preceding trauma.

Our case suggests that there may be a close relationship between centrifugal and progressive lipodystrophy, as discussed by Imamura *et al.*[1] However, most reported cases of both disorders have distinct features as the Table shows. In addition, systemic involvement such as hypocomplementaemia[6] and glomerulonephritis[7] is frequently associated with progressive lipodystrophy, whereas no such associations have been demonstrated in centrifugal lipodystrophy. Therefore, these two disorders appear to constitute different disease entities. Furthermore, we consider that the presence of an erythematous border is a prerequisite for the diagnosis of centrifugal lipodystrophy.

Acknowledgments

We wish to thank Dr Masato Kawabata (Department of Clinical Pathology, School of Medicine, Nihon University, Japan) for performing the serological test for *Borrelia burgdorferi* and Mrs Akie Koseki for her technical assistance.

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British Journal of Dermatology (1992) **127**, 411–416.

Elephantiasis nostras verrucosa: beneficial effect of oral etretinate therapy

CH.C.ZOUBOULIS, S.BICZÓ,* H.GOLLNICK, H.-J.REUPKE, G.RINCK, M.SZABÓ,* J.FEKETE† AND C.E.ORFANOS

Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Germany
*Departments of *Dermatology and †Pathology, 'Kaposi Mór' County Hospital, Kaposvár, Hungary*

Accepted for publication 1 March 1992

Summary

Elephantiasis nostras verrucosa is characterized by chronic secondary, non-filarial lymphoedema due to recurrent lymphangitis, dermal fibrosis, and epidermal changes consisting of hyperkeratotic, verrucous and papillomatous lesions. Histologically, there is pseudoepitheliomatous hyperplasia. Therapeutic efforts should aim to reduce lymph stasis, which will also lead to improvement of the cutaneous changes. In this study, rapid disappearance of the hyperkeratotic and verrucous lesions, remarkable flattening of the papillomatous nodules and improvement of lymphoedema occurred in three obese patients treated with etretinate in an initial dose of 0·6–0·75 mg/kg/day for 4–6 weeks. Monitoring of plasma concentrations of etretinate, acitretin and 13-*cis*-acitretin by HPLC revealed sufficient short-time absorption (4 h) and bioavailability of the drug (30 days; two out of three patients). Long-term maintenance therapy in one patient produced a remarkable improvement in the lymphoedema; another patient relapsed after discontinuation of the etretinate and responded again after this was reintroduced. In the third patient treatment was withdrawn because of an increase in triglycerides, but improvement persisted 6 months later. The clinical side-effects of oral retinoid therapy were moderate and well tolerated.

The term elephantiasis nostras verrucosa has been used to describe chronic secondary, non-filarial lymphoedema, with cutaneous changes consisting of dermal fibrosis and hyperkeratotic, verrucous and papillomatous lesions showing pseudoepitheliomatous hyperplasia.[1–8] This condition has been reported under a variety of other names, including elephantiasis nostras,[9–15] elephantiasis verrucosa,[16–18] elephantiasis crurum papillaris et verrucosa,[19] lymphostatic papillomatosis cutis,[20,21] lymphostatic verrucosa,[22] papillomatosis cutis verrucosa,[21] pachydermia vegetans,[23] and mossy foot and leg.[1,4,13]

The disease is uncommon, and no standard treatment exists for the cutaneous manifestations. In this report we describe three obese patients with elephantiasis nostras verrucosa who were successfully treated with oral etretinate.

Case reports

Case 1

A 98-year-old obese woman with cardiac and renal insufficiency was admitted to hospital because of fever and painful erythematous swelling of both legs (left> right) with bulla formation. Erysipelas was diagnosed. In addition, she had secondary chronic lymphoedema of both legs, with a cobblestone-like skin surface due to malodorous, hyperpigmented, hyperkeratotic lesions. There were large verrucous nodules on the feet, and onychogryphosis (Fig. 1a). Histological examination of the verrucous lesions showed pseudoepitheliomatous hyperplasia and excluded squamous cell carcinoma (Fig. 1b). After antibiotic treatment of the erysipelas and stabilization of the cardiac and renal status, oral etretinate (0·75 mg/kg/day) was introduced for 20 days, followed by 0·5 mg/kg/day for 10 days and 0·25 mg/kg/day for a further 15 days. Plasma concentrations of etretinate and its metabolites, acitretin and 13-*cis*-acitretin, were measured 4 and 24 h after drug administration by reverse-phase HPLC, and all values were found to be within therapeutic levels (Fig. 2). Salicylic

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Correspondence: Dr Ch.C.Zouboulis, Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Hindenburgdamm 30, W-1000 Berlin 45, Germany.

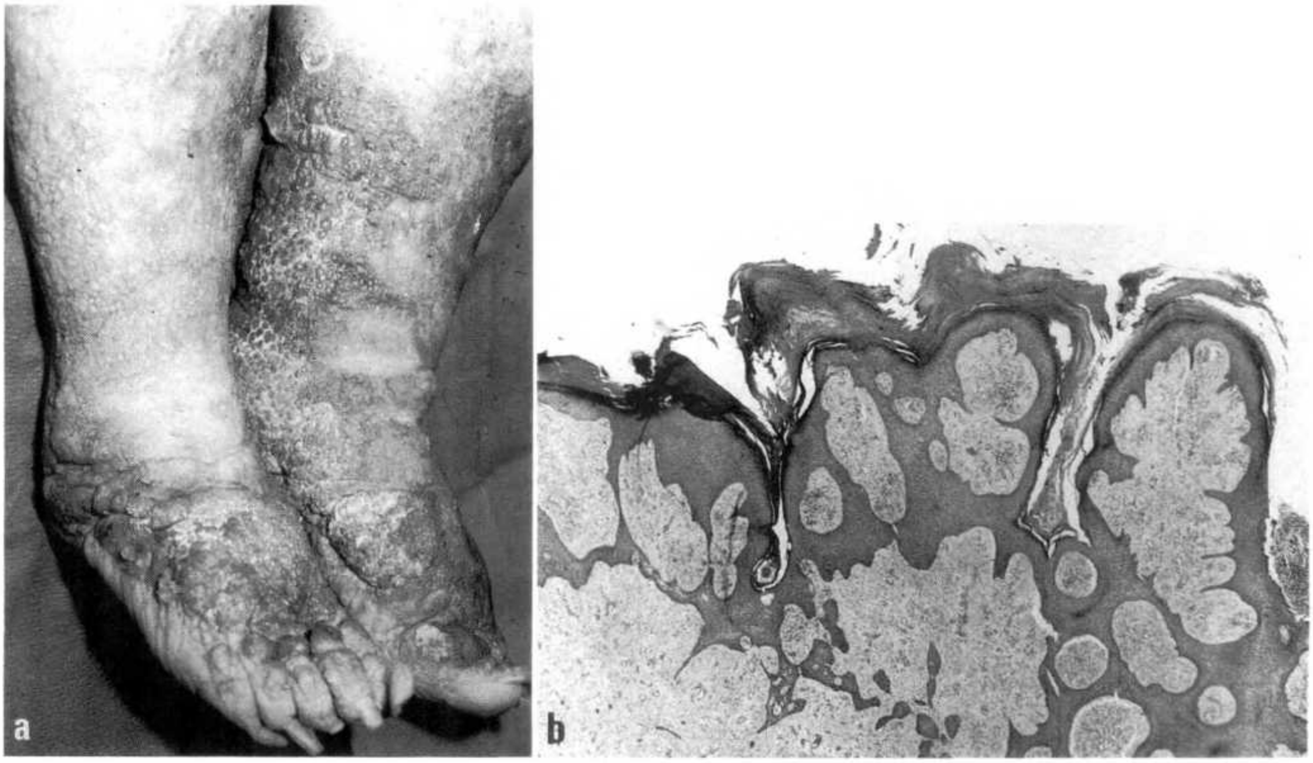

Figure 1. (a) Elephantiasis nostras verrucosa before treatment (Case 1). (b) Histology shows irregular acanthosis with focal hypergranulosis, marked orthohyperkeratosis and elongated rete ridges. Little dysplasia or mitotic activity is seen and the basal cell layer appears intact. The dermis shows a chronic inflammatory infiltrate and dilated capillaries (haematoxylin and eosin × 23).

acid ointment (5%) was applied topically for a few days. Etretinate treatment resulted in disappearance of the verrucous masses and remarkable flattening of all hyperkeratotic lesions (Fig. 3a). The histology almost reverted to normal (Fig. 3b). Because of an increase of serum triglycerides (from 2·76 mmol/l to 10·78 mmol/l; normal value ≤ 3·00 mmol/l), the treatment was discontinued after 45 days. There was no relapse during the following 6 months. Liver function tests and serum cholesterol remained normal during treatment. Cheilitis was the only mucocutaneous side-effect observed.

Case 2

An 82-year-old obese woman presented with secondary chronic lymphoedema of the right leg following recurrent erysipelas. There were malodorous, hyperkeratotic lesions on the right leg, verrucous nodules on the toes of the right foot, and a papillomatous tumour below the medial malleolus, on the lower part of a chronic venous ulcer. Histological examination of both the hyperkeratotic lesions and the malleolar tumour showed pseudoepitheliomatous hyperplasia. She was treated with oral etretinate (0·75 mg/kg/day) for 1 month, 0·5 mg/kg/day for a further 5 months, and subsequently 0·25 mg/kg/day as long-term maintenance therapy. In addition, she was treated with spironolactone 200 mg/day and

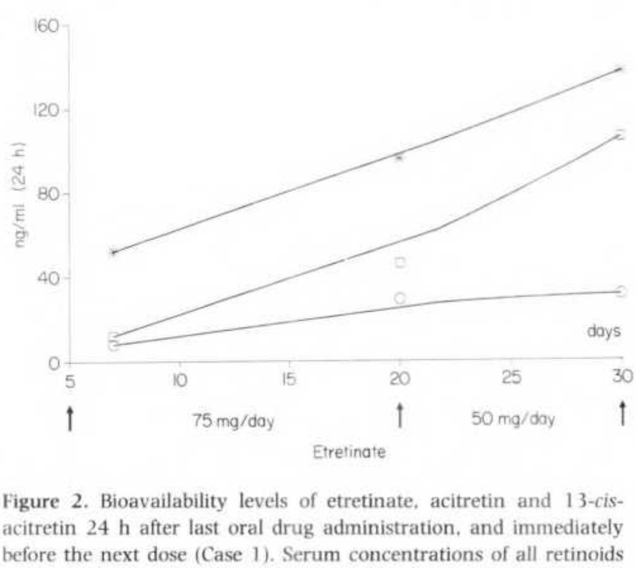

Figure 2. Bioavailability levels of etretinate, acitretin and 13-*cis*-acitretin 24 h after last oral drug administration, and immediately before the next dose (Case 1). Serum concentrations of all retinoids assessed were low after 20 days, but achieved therapeutic levels[45] after 30 days. —□—, etretinate; —○—, acitretin; —*—, 13-*cis*-acitretin.

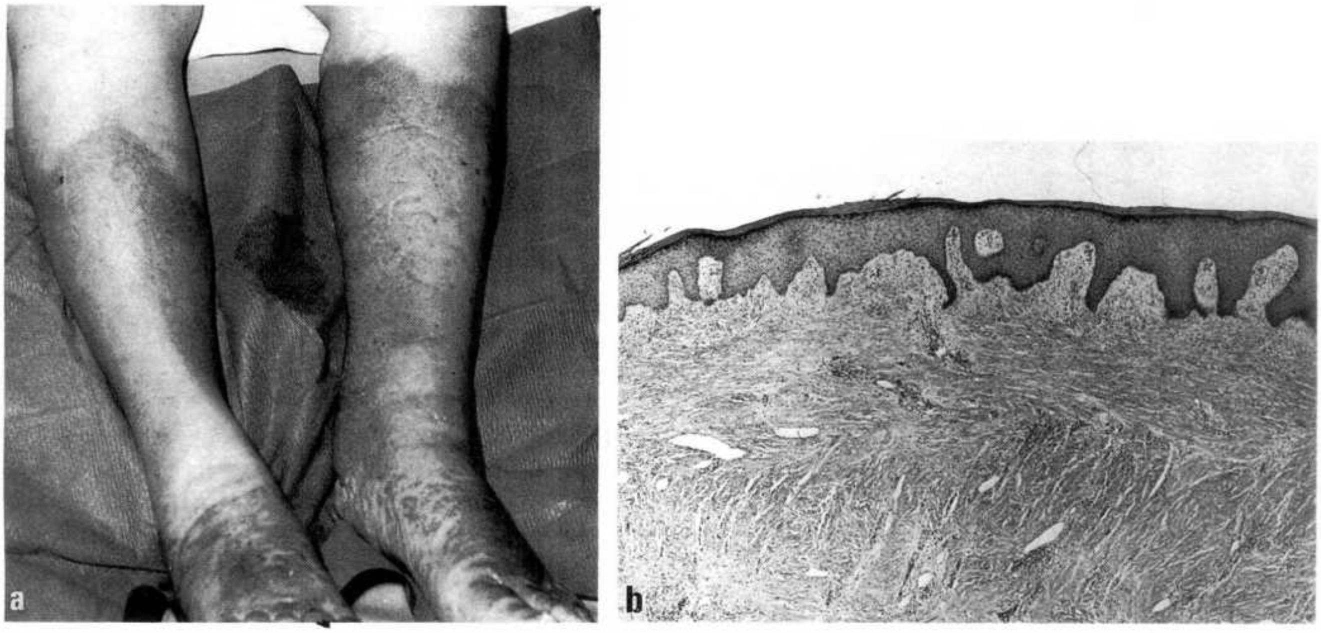

Figure 3. (a) Elephantiasis nostras verrucosa after 5 weeks of etretinate treatment (Case 1). (b) Histology shows an acanthotic epidermis, absence of hyperkeratosis, and shortened rete ridges. Dermal inflammation and the number of capillaries are reduced (haematoxylin and eosin × 34).

topical 5% salicylic acid ointment. This treatment produced marked resolution of the verrucous lesions. Plasma concentrations of etretinate, acitretin and 13-*cis*-acitretin and their 24-h profile after 3 weeks of treatment were assessed by reverse-phase HPLC, and were found to be within therapeutic levels (Fig. 4). Leg and foot circumferences were measured before and during treatment and confirmed the beneficial effect (Fig. 5). Liver function tests, serum cholesterol and triglycerides remained normal. Cheilitis and palmoplantar desquamation were the only mucocutaneous side-effects.

Case 3

A 67-year-old obese woman suffering from heart failure was admitted to hospital because of progressive development of malodorous, verrucous, cobblestone-like lesions on both legs and feet for 3 years. There was persistent oedema of both legs (Fig. 6a). Histological examination of the verrucous skin showed pseudoepitheliomatous hyperplasia. After treatment of her heart failure, she was given oral etretinate (0·6 mg/kg/day) for 6 weeks. The dose was subsequently reduced to 0·2 mg/kg/day for an additional 10 days. Salicylic acid ointment (5%) was applied topically. This treatment produced a marked reduction of hyperkeratosis, and the verrucous lesions resolved (Fig. 6b). She was discharged from hospital and intermittent maintenance treatment with etretinate was advised, but was not given. Ten weeks later she presented with new hyperkeratotic lesions around the ankles. She was given etretinate (0·5 mg/kg/day) for 2 weeks and remarkable improvement occurred. Liver function tests, serum cholesterol and triglycerides were normal before, during and after treatment. There were no clinical side-effects.

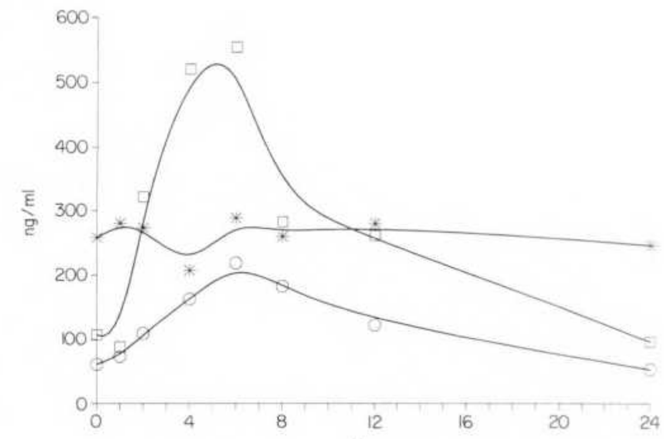

Figure 4. Full 24 h pharmacokinetic profiles of etretinate, acitretin and 13-*cis*-acitretin plasma concentrations after 3 weeks of treatment (Case 2). Maximal peaks of etretinate and acitretin are seen 4–6 h after drug administration. The levels of 13-*cis*-acitretin remain stable during the entire 24 h period. —□—, etretinate; —○—, acitretin; —*—, 13-*cis*-acitretin.

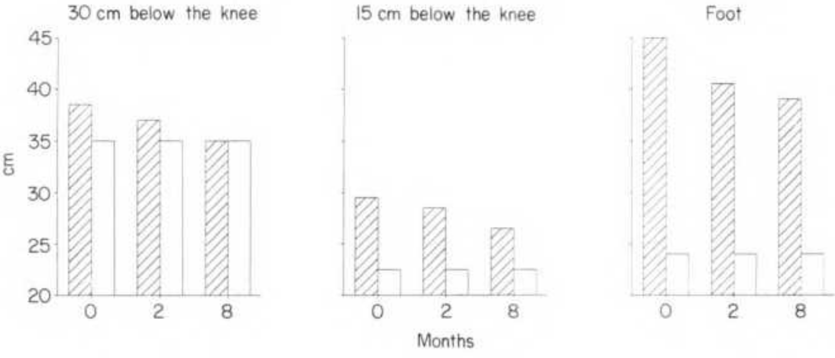

Figure 5. Leg and foot circumferences before and during treatment with etretinate (Case 2). There was marked, sustained reduction in circumference measurements on the affected right leg and foot. No changes were noted on the healthy left limb. ■, right; □, left.

Discussion

Lymphatic vessels are the major escape routes for excess proteins and fluid in tissues, being fundamentally important in regulating cell hydration and osmosis.[6,24,25] Functional incapacity of the lymphatic and the tissue proteolytic systems to remove excess plasma proteins may give rise to lymphoedema.[6,26,27] A high interstitial protein concentration then retains fluid in tissues as a result of osmotic forces, and causes chronic inflammatory changes leading to fibrosis and epidermal hyperplasia. Thus, chronic peripheral oedema due to lymph stasis may produce thickened skin with hyperkeratotic verrucous and papillomatous lesions, and these changes are known as elephantiasis.[7,28]

Elephantiasis nostras of the glabrous or the verrucous type is the result of recurrent attacks of lymphangitis.[2,5,8] It is most frequently manifest on the leg, but other parts of the body such as the scrotum, abdominal wall, face and ears may also be involved.[2,3,15,29] The disease must be distinguished from other types of secondary elephantiasis, for example those due to tumour-associated lymphoedema and filariasis.[1,5,6] Mixed venous/lymphatic oedema caused by venous hypertension or chronic cardiac failure can also induce or promote elephantiasis, as seen in Case 3.[11,13,14,18]

Elephantiasis nostras verrucosa and papillomatosis cutis carcinoides, described by Gottron,[30,31] are closely related. The latter is a rare condition consisting of single or multiple verrucous, partly ulcerated tumours on chronically irritated oedematous skin.[30,32] The two entities show common histological findings of pseudoepitheliomatous hyperplasia,[6,18,31] and their distinctive clinical features may represent different stages of the same disease. In contrast with the benign course of elephantiasis nostras verrucosa, papillomatosis cutis

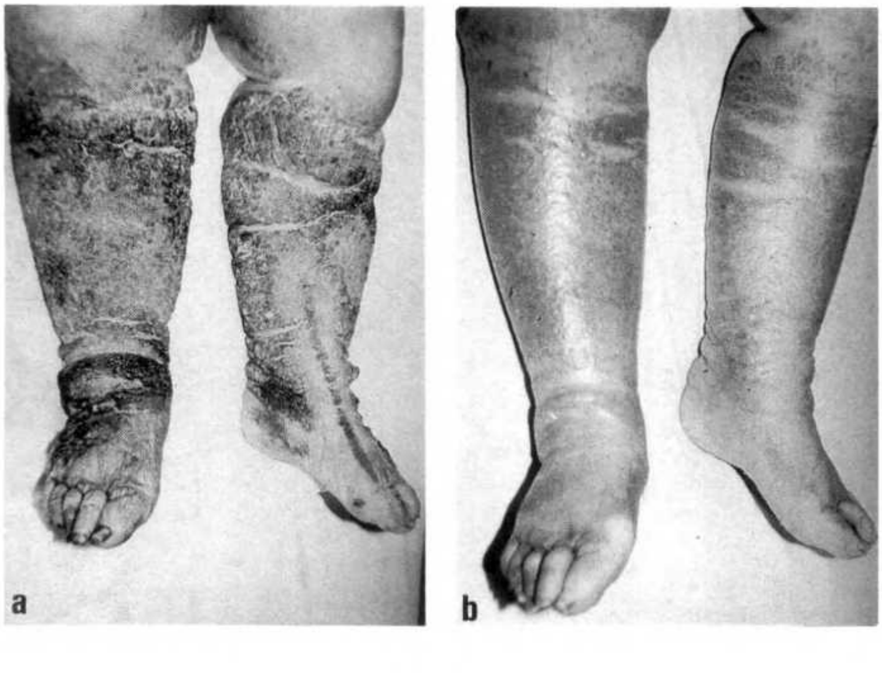

Figure 6. Elephantiasis nostras verrucosa (Case 3): (a) before treatment, (b) after 6 weeks of treatment with etretinate.

carcinoides may undergo malignant transformation.[33-35]

Treatment of elephantiasis nostras verrucosa is difficult. Therapeutic efforts with a combination of manual and mechanical massage, compression with elastic bandages, or intermittent use of pneumatic stockings, are directed at reducing the lymphoedema, whereby secondary improvement of skin lesions may occur.[4,6,18,26,28,36] In addition, antibiotics and local wound care are occasionally necessary. Debridement of the verrucous lesions[13,18,37] and topical keratolytic agents[21] have been shown to be effective in the treatment of the concomitant hyperkeratotic condition. Leg elevation and external pressure were not utilized in the management of our patients, in order to better evaluate the effect of retinoid therapy.

Systemic retinoids, especially etretinate, are well established in the treatment of diseases characterized by epidermal hyperproliferation, and in disorders of keratinization.[38] They affect keratinocytes in many ways and induce loosening and thinning of hyperkeratotic stratum corneum.[39] The epidermal lesions in our patients probably responded to treatment with oral etretinate because of this mechanism of action. Two other cases with similar disorders, effectively treated with etretinate and 3% retinoid ointment, have been reported.[12,40] In addition, retinoids act in a variety of ways on fibroblast function, possibly induce collagen degradation, and have beneficial effects on fibrosis.[41,42] They also possess anti-inflammatory activity.[43] These properties may have contributed to the therapeutic effect of etretinate on the dermal changes in our patients.

Reverse-phase HPLC has been established in our laboratory for routine drug monitoring[44] and the values obtained here showed no alterations of etretinate maximum levels and bioavailability in two out of three patients, in spite of their obesity. Plasma retinoid concentrations indicated sufficient absorption maxima 4 and 24 h after drug administration. The values obtained 30 days after commencing oral medication correlated well with the clinical response. In addition, the pharmacokinetic retinoid profiles over 24 h corresponded with those in psoriatic patients treated with similar daily doses of etretinate.[45]

Mucocutaneous side-effects were moderate, but marked elevation of serum triglycerides, as observed previously in 27% of all patients treated with etretinate,[46] was the reason for discontinuation of therapy in one patient.

Our observations indicate that elephantiasis nostras verrucosa may represent a reasonable new indication for etretinate therapy.

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British Journal of Dermatology (1992) 127, 417–441

SOCIETY PROCEEDINGS

British Society for Investigative Dermatology Annual Meeting, Sheffield, 24–25 September 1992

Papers

IMMUNOLOCALISATION OF THE p53 TUMOUR SUPPRESSOR GENE PRODUCT IN NORMAL AND DISEASED HUMAN EPIDERMIS. M J Edwards, R Thomas, C M Mills, P J Dykes and R Marks. Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN.

The p53 tumour suppressor gene product is a nuclear phosphoprotein which is thought to play a central role in the negative regulation of cell growth and division The wild type p53 protein can inhibit DNA synthesis and block cell growth in the G1 phase of the cell cycle. However, studies into the function of the wild type p53 in growth control have proved difficult, due in part to the proteins short half life in cultured mammalian cells. We have investigated the expression of the wild type p53 gene product in normal human epidermis and in the hyperproliferating epidermis of psoriasis lesions Cryostat sections (4μm) of normal and psoriatic human skin were exposed for 30 minutes at 37°C to one of the following -a) an IgG monoclonal antibody control, b) monoclonal antibody specific for wild type p53 protein, c) monoclonal antibody with specificity towards mutant p53 and denatured normal p53 protein The primary antibody was then localised using a two step immunoperoxidase procedure The molecular weight of the p53 gene product was confirmed by the immunoprecipitation of [^{35}S] methionine labelled proteins from skin explants Wild type p53 protein was detected within the nuclei of epidermal cells from both normal and psoriatic epidermis. However, higher levels of wild type p53 were detected in resolving psoriasis lesions when compared with normal epidermis Mutant p53 was not detected in normal human epidermis. Mutant p53 protein was detected in the nuclei and cytoplasm of resolving psoriasis lesions. Our data suggests that the p53 tumour suppressor gene product is being expressed at high levels in resolving psoriasis lesions Two explanations can be proposed for the presence of mutant p53 in psoriasis lesions. Firstly, a mutation may have occured in the wild type p53 gene However, a more likely explanation is that the mutant p53 monoclonal antibody has recognised a denatured form of the wild type p53 protein which in turn reflects an increased rate of wild type p53 protein turnover in these lesions.

P53 MUTATION IS AN EARLY EVENT IN EPITHELIAL CARCINOGENESIS: DETECTION OF A CODON 248 POINT MUTATION IN BOWEN'S DISEASE.

C.Campbell, Y.Ro, A.G.Quinn, B.Angus and J.L.Rees, Department of Dermatology, University of Newcastle upon Tyne.

P53 is a tumour suppressor gene and mutations in this gene and/or deletions are the commonest genetic events yet identified in human neoplasia. We show that abnormal immuno-staining for p53 which implies mutation of the gene is present in up to 50% of squamous cell carcinomas and basal cell carcinomas and 20-30% of pre-invasive lesions, such as Bowen's disease and actinic keratosis. Other groups using DNA sequencing and immunocytochemistry have failed to detect mutations in pre-invasive lesions, arguing that the immuno-staining observed is non-specific. We have therefore carried out further immunocytochemistry studies using not only the monoclonal antibody DO7 on frozen and paraffin embedded specimens, but also with CM1, a polyclonal antibody, and demonstrated abnormal staining in 30% of preinvasive actinic keratosis and cases of Bowen's disease (n=30). We have then gone on to further examine cases of Bowen's disease which show positive immuno-staining, and, although to date we have only sequenced exon 7, we have identified 1 point mutation, a C-T transversion at codon 248 in a case of Bowen's disease. Given the number of exons in which mutations may occur, our results are in keeping with our previous immunocytochemistry data, suggesting that p53 is an early marker of genetic change in epithelial carcinogenesis and will therefore lend itself to further molecular epidemiological studies of the role of ultraviolet light in squamous cell carcinoma.

ABERRANT EXPRESSION OF P53 TUMOUR SUPPRESSOR PROTEIN IN CUTANEOUS SQUAMOUS CELL CARCINOMAS FROM RENAL ALLOGRAFT RECIPIENTS - EVIDENCE AGAINST AN ONCOGENIC ROLE FOR HUMAN PAPILLOMAVIRUS? JM McGregor, C C-W Yu*, DA Levison*, DM MacDonald. Depts of Dermatology and Histopathology*, Guy's Hospital, London SE1 9RT.

Compelling evidence suggests a role for human papillomavirus (HPV) in the aetiology of both verrucous keratoses and squamous cell carcinomas from renal allograft recipients but not of skin cancers from the general population. A possible mechanism is the functional inactivation of p53 tumour suppressor protein by HPV-encoded oncoproteins, as occurs in cervical tumorigenesis. To examine this hypothesis, an immunohistochemical technique was employed using CM-1, an antiserum raised against recombinant human p53 protein, to determine expression of the p53 tumour suppressor protein in verrucous keratoses, solar keratoses and squamous cell carcinomas from both immunosuppressed and non-immunosuppressed patients. P53 immunostaining, not found in normal cells, is detectable in diverse tumours because the mutant protein assumes a more stable configuration. In HPV-associated cervical tumours however, p53 expression is not seen since functional inactivation of p53 occurs, not through mutation, but through enhanced degradation of the protein

P53 expression was found in 40% (6/15) of verrucous keratoses and 66% (8/12) of squamous cell carcinomas from renal allograft recipients compared with 55% (5/9) of solar keratoses and 56% (7/12) of squamous cell carcinomas from non-immunosuppressed patients. A similar prevalence of p53 immunostaining observed in tumours from both immunosuppressed and non-immunosuppressed patients suggests that p53 gene mutation, rather than functional inactivation of p53 by viral oncoproteins. is likely to play an important role in transplant squamous cell tumorigenesis If HPV has a role in the aetiology of transplant skin cancers, a mechanism distinct from that found in HPV-associated cervical neoplasia would have to be postulated.

PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) DECREASES IN NORMAL KERATINOCYTES (NKS) WITH DIFFERENTIATION STIMULI BUT NOT IN IMMORTAL HUMAN PAPILLOMA VIRUS (HPV) TRANSFORMED CELLS.

K.T. Jones, [1]K.H. Vousden and G.R. Sharpe, Dept. of Dermatology, Univ. of Liverpool, and [1]Ludwig Institute for Cancer Research, London.

PCNA (cyclin) increases the processivity of DNA polymerase δ. The effects of differentiation agents (high calcium, vitamin D_3 ($1\alpha,25(OH)_2\ D_3$) and the phorbol ester TPA) on PCNA levels in cultured NKs and differentiation resistant, HPV transfected cells have been investigated.

Keratinocytes were grown in the serum free medium MCDB 153 with the addition of 1% bovine hypothalamic extract for HPV cells. Cultures were incubated for 48h in medium containing 70µM Ca^{++}, 2mM Ca^{++}, 20nM TPA, or 1µM D_3. Cells were fixed with 1:1 methanol:acetone and incubated at room temperature (30min) with 1:50 mouse anti-PCNA monoclonal antibody (PC10 Novacastra) after washing in phosphate buffered saline and 10% rabbit serum (10min) to block non-specific binding. The secondary antibody was fluorescein (1:30) or rhodamine (1:50) conjugated rabbit anti-mouse immunoglobulin (Dako).

PCNA staining was confined to the nucleus and categorised as shown in the table. All observations were repeated 2-10 times with 200-1000 cells counted. PCNA staining in (a) NKs (b) HPV cells (percentage of total ± SEM).

	strong		diffuse		weak/none	
	a	b	a	b	a	b
Ca^{++} (70µM)	44±1	44±5	39±2	47±4	17±1	9±1
Ca^{++} (2mM)	13±3	45±6	23±4	41±7	64±4	14±1
D_3 (1µM)	9±6	27±6	13±2	47±8	78±5	26±4
TPA (20nM)	3±2	34±3	4±1	37±2	93±3	29±1

PCNA expression was greatly reduced by high Ca^{++}, TPA and $1\alpha,25\ (OH)_2D_3$ in NKs but not in HPV cells. It is concluded that PCNA levels are decreased when keratinocytes differentiate, but are maintained in differentiation resistant cells which continue to cycle.

HA-RAS CODON 12 MUTATIONS DO NOT PLAY AN IMPORTANT ROLE IN THE MAJORITY OF NON-MELANOMA SKIN CANCERS.

C.Campbell, A.G.Quinn, J.L Rees. University Department of Dermatology, Newcastle-upon-Tyne.

Although non-melanoma skin cancer is the most common human malignancy, the molecular events underlying the progression to neoplasia are poorly understood. Initial studies reported low frequencies of ras mutations in non-melanoma skin tumours. However, a recent study from Texas reported a high frequency of mutations at the second position of Ha-ras codon 12 in both basal cell carcinomas and squamous cell carcinomas, and prompted us to re-examine the role of Ha-ras codon 12 mutations as a possible early step in skin carcinogenesis. We required a sensitive method, capable of detecting mutations when present in only a low percentage of cells. Using a recently described technique based on polymerase chain reaction followed by codon 12 mutant specific restriction fragment length polymorphism, we demonstrated with a positive control (bladder EJ cell line known to contain a G->T transition at Ha-ras codon 12) that this method was capable of detecting mutations at this codon when only 4% of the total alleles are mutated. On subsequent analysis of 40 BCCs, 12 SCCs and 12 Bowen's disease, we were surprised to find no mutations in any tumour. The use of positive and negative controls and confirmatory sequencing allow us to be confident that our results accurately reflect the incidence of Ha-ras codon 12 mutations, and we conclude that these mutations are rare events in non-melanoma skin tumours in the United Kingdom. Differences in previously reported rates may therefore relate to technical artefacts or differences in the molecular epidemiology between low and high sun exposure areas.

MINISATELLITE ANALYSIS DETECTS COMPLEX SOMATIC DNA CHANGES IN INTRAEPIDERMAL AND INVASIVE CUTANEOUS SQUAMOUS CELL CARCINOMAS.

A.G Quinn, C.Campbell, J.L.Rees, Department of Dermatology, University of Newcastle upon Tyne

DNA minisatellite probes which can detect a large number of autosomal loci dispersed throughout the human genome were used to examine constitutional and tumour DNA of patients with Bowen's disease and cutaneous squamous cell carcinomas. Southern blots of tumour and normal DNA digested with HinfI were hybridised with riboprobes 19.6 and 18.15 derived from minisatellite probes 33.6 and 33.15. Samples showing differences in the banding pattern between normal and tumour DNA were digested with Alu I and Hae III to further characterise the observed changes.

Changes in the banding pattern were observed with Hinf digests in 6 of 15 cases of Bowen's disease and 4 of 6 squamous carcinomas and consisted of changes in band intensity, loss of bands or the presence of novel bands. Digestion of DNA with methylation resistant endonucleases indicated that some of the observed changes are due to tumour specific changes in methylation as the constitutional DNA was extracted from normal skin. Unexpectedly, in one individual with multiple SCC's, similar changes in banding pattern were observed in different tumours in Hinf I restricted DNA.

These results demonstrate that cutaneous neoplasms defined as 'early' in clinico-pathological terms show complex genomic changes at the molecular level. The observation of consistent changes in banding pattern in HinfI digests of multiple tumours from the same individual suggests that changes in methylation may be specific and therefore possibly important in cutaneous carcinogenesis.

IN VIVO LOSS OF TELOMERIC REPEATS IN HUMAN BASAL AND SQUAMOUS CELL CARCINOMA AND WITH AGEING.

L.J.Wainwright, P.G.Middleton*, J.L.Rees, Departments of Dermatology and Medicine*, University of Newcastle upon Tyne.

Telomeres are specialized regions of DNA at the terminal end of linear chromosomes which protect the end from degradation or fusion and allow its complete replication. Telomeric DNA consists of repeats of a simple G-rich sequence; in humans the majority and most distal repeats are of the form TTAGGG. Previous results have suggested that telomeres may play a role in the aetiology of tumours (Hastie et al. Nature 346:866-868,1990). In the present experiments, we have examined the length and number of tandem repeat arrays of the telomeres from different patients with basal and squamous cell carcinoma. DNA was prepared by standard procedures from the skin samples of different individuals and loaded on to an agarose gel. Southern blots of DNA were hybridised with a ^{32}P-labelled (TTAGGG)$_6$ probe at 65°C with a final filter wash of 1 X SSC, 0.1% SDS at 65°C. The resulting autoradiographs show patients with basal and squamous cell carcinomas to have a reduced telomere length than the paired normal skin sample.

DNA was also prepared from dermal fibroblasts and epidermal keratinocytes. The resulting autoradiographs show a significant reduction in telomere length and intensity between keratinocytes and fibroblasts. This was correlated with an observed reduction in telomere length and intensity between epidermis and dermis. It has previously been proposed that a decrease in the length of telomeres with the successive rounds of DNA replication that accompany mitotic division could play a causal role in the ageing process. In this experiment we set out to show that the tandem repeat arrays of the telomere decreases with age. Normal skin was taken from 9 individuals, with ages ranging from 3-88 years old. Both telomeric length and quantity of repeat sequences were found to decrease with age. Thus, loss of telomeric sequences may lead to senescence or just be an associated effect of ageing.

QUANTITATIVE EVALUATION OF MHC CLASS II (Ia) EXPRESSION BY LANGERHANS CELLS FOLLOWING TOPICAL APPLICATION OF CHEMICAL ALLERGENS.

M Cumberbatch, R J Dearman, [1]D A Basketter and I Kimber, ICI Central Toxicology Laboratory, Alderley Park, Cheshire and [1]Unilever Environmental Safety Laboratory, Sharnbrook, Bedfordshire.

It has been reported recently that topical exposure of mice to a variety of contact allergens, including trinitrochlorobenzene, dinitrofluorobenzene, oxazolone and cinnamic aldehyde, results in a significant augmentation of major histocompatibility complex (MHC) class II (Ia) expression by Langerhans cells (LC) (Aiba S, Katz SI. Phenotypic and functional characteristics of in vivo-activated Langerhans cells. J Immunol. 1990; 145 · 2791-6). In the present study we have measured alterations in Ia expression by LC following epicutaneous application of two sensitizing chemicals, 2,4-dinitrochlorobenzene (DNCB) and trimellitic anhydride (TMA). Although both are known to cause contact hypersensitivity, only TMA is able to induce respiratory hypersensitivity in man, and only TMA provokes IgE antibody responses in mice (Dearman RJ, Kimber I. Differential stimulation of immune function by respiratory and contact chemical allergens. Immunology 1991; 72 : 563-70).

In the present investigations LC were isolated from the local epidermis of mice following topical exposure to concentrations of DNCB (1%) and TMA (50%) which resulted in equivalent levels of contact hypersensitivity. Flow cytometric analyses revealed that treatment with DNCB, but not with TMA or with vehicle alone, caused a time-related increase in Ia expression by LC, reaching 160% of control values within 18 hours of exposure. Concurrent measurement of forward-angle light scatter indicated that the increased expression of Ia by LC prepared from skin treated with DNCB was not associated with alterations in cell size.

These studies demonstrate that not all chemicals which have the potential to cause contact hypersensitivity effect an increase in Ia expression by LC and that chemical allergens which induce qualitatively different immune responses also exert divergent effects on LC in situ.

TITLE: "LANGERHANS CELL HISTIOCYTOSIS CELLS" ARE DEFECTIVE ALLOANTIGEN-PRESENTING CELLS.

R.C.H.Yu, M. Alaibac and A.C. Chu. Unit of Dermatology, Royal Postgraduate Medical School, London W12 0NN.

Although it is well recognised that lesional Langerhans cell histiocytosis cells (LCH cells) express most of the enzyme histochemical and immunophenotypic characteristics of normal epidermal Langerhans cells (LC), the functional capacity of LCH cells as antigen-presenting cells has rarely been examined. In the present study, we examined the alloantigen-presenting activity of LCH cells derived from lesional sites of three patients affected by LCH. Lesional LCH cells from three different tissues (skin, lymph node and gum respectively) of these patients were enriched using three different methods: dispase enzyme digestion, fluorescein-activated cell sorting and negative selection with immunomagnetic beads. The LCH cells were used as stimulator cells in a conventional 6-day allogeneic mixed cell reaction, using peripheral blood mononuclear cells from 3 healthy volunteers as responders. Involved and non-involved epidermis from patient 1 and normal surgical epidermis were used as controls.

LCH cells from involved dermis of patient 1 and lymph node of patients 2 showed minimal functional activity, and that from gum tissue of patient 3 showed reduced activity on a per cell basis when compared to normal epidermal LC. Epidermal LC from non-involved skin of patient 1 demonstrated normal functional activity, and epidermal LC overlying the involved skin showed an augmented activity when compared to the positive controls.

Mean +/-SD of [^{3}H]-thymidine incoporation in decays per minute of various stimulator cell populations at 1000 CD1a positive cells per well.

Patients	Tissues	Counts (DPM)
1	Lesional dermal cells	4230 +/-916
1	Normal epidermal cells	55102 +/-13793
1	Lesional epidermal cells	120261+/-26404
2	Lymph node	1500 +/-440
3	gum	47863 +/-7866
Normal control epidermal cells		85011 +/-9173

Our findings strongly suggest that LCH represents a disease in which functionally defective LC accumulate and/or proliferate in various tissues, and that the defect appears to be localised to the lesional LCH cells and does not affect the entire LC population.

THE EXPRESSION OF FcϵRII/CD23 RECEPTORS ON MACROPHAGE SUBSETS IN ATOPIC DERMATITIS.

C. Buckley C. Ivison L. W. Poulter* M.H.A. Rustin
Dept of Dermatology and Immunology* Royal Free Hospital and School of Medicine*, London.

We have previously reported that lesional skin of patients with atopic dermatitis (AD) has an increased number of RFD1+ dendritic cells and CD1+ Langerhans cells (1). Furthermore following patch testing with aeroallergens there was a change in the expression of the low affinity IgE receptor (CD23) with downregulation on RFD7+ cells and an increased expression on RFD1+ and CD1+ cells. The present study has assessed the expression of CD23 during the maturation and differentiation of peripheral blood monocytes taken from patients with AD.

Peripheral blood monocytes were isolated from 10 patients with AD, 8 patients with atopic asthma and 6 non-atopic controls. The cells were cultured in RPMI and 10% fetal calf serum and cells harvested at 0, 5 and 7 days. Cytospin preparations were prepared and stained with MoAbs to mature macrophages (RFD7+), dendritic cells (RFD1+) and CD23.

There was a more rapid appearance of monocytes expressing RFD1+ and RFD7+ from patients with AD ($p < 0.05$) but by day 7 there was no significant difference in the increased expression of these phenotypes in all three groups. There was a significantly greater expression of CD23+RFD1+ and CD23+ RFD7+ cells at day 7 from AD patients (90%+/-5 and 90%+/-8 respectively) versus the controls (40%+/-8 and 38%+/-11 respectively) ($p < 0.001$). However the total percentage of cells expressing CD23 did not change during the 7 day culture. This study has shown a more rapid maturation of monocytes from patients with AD and an increased number of CD23+ cells that have antigen presenting capacity.

1. Buckley C, Ivison C, Poulter L.W. Rustin M.H.A.

FCϵ R11/CD23 Receptor Distribution in Patch Test Reactions to aeroallergens in Atopic Dermatitis. J. Invest Dermatol. 1992 in press.

INFLUENCE OF PIGMENTATION AND ILLUMINATION ON THE PERCEPTION OF ERYTHEMA

B L Diffey, Regional Medical Physics Department, Dryburn Hospital, Durham

The visual assessment of erythema induced by ultraviolet radiation (UVR) involves a comparison of the irradiated site with surrounding non-irradiated skin. A threshold response, such as the minimal erythema dose (MED), will be recorded when the observer perceives a just noticeable difference between these two areas of skin. It has been appreciated for some time that there is a relationship between darkness of complexion and erythema threshold. The purpose of the present study was to investigate the relationship quantitatively and also to examine how the spectral composition of the illuminating source influenced the assessment of the MED.

The untanned skin on the inner aspect of the lower forearm of a Caucasian male was irradiated with a series of increasing UVB exposures. 24h after irradiation 13 observers examined independently the irradiated sites under two different illuminating sources: an incandescent tungsten light blub and white fluorescent lamps. For each light source the observers were asked to indicate whether or not each irradiated site appeared erythematous compared with surrounding skin. A number of plastic filters of varying shades of brown ranging from very pale tan to brown/black (to simulate differing degrees of epidermal pigmentation) were placed individually over the skin and each observer asked again to indicate the presence or absence of erythema.

For each shade of brown filter (including no filter) a chi-squared test indicated no difference ($P > 0.9$) in the perception of erythema when the skin was viewed under either light source. However, as the absorbance of the filter increased (equivalent to increasing melanin pigmentation), so did the ultraviolet dose at which erythema became generally perceptible. This is shown below where the MEDs (defined as the lowest dose at which at least 7 of the 13 observers indicated a difference between the irradiated site and surrounding skin) observed in "dark tanned" and "brown/black" skin were about 20% and > 80% higher, respectively, than in white or mildly tanned skin, despite the fact that identical vasodilation had occurred.

Filter	Equivalent Pigmentation	MED mJ/cm²	
		Tungsten lamp	Fluorescent lamp
None	White, untanned skin	35.4	35.4
1	Pale tan	35.4	35.4
2	Pale tan	35.4	35.4
3	Mild tan	35.4	35.4
4	Dark tan	42.9	39.0
5	Dark tan	47.2	47.2
6	Brown/Black	62.8	>62.8
7	Brown/Black	>62.8	>62.8

In conclusion, illuminating skin with either incandescent or fluorescent lighting at the same illuminance did not affect the determination of the MED. Likewise the range of pigmentation that may occur in Caucasian skin had little or no effect on the perception of the MED compared with white, untanned skin. However the results indicated that the doses required in Negro skin to achieve comparable vasodilation associated with an MED in white skin are probably a factor of two or so less than the visually assessed MED in black skin. This suggests that the usually observed MED may over-estimate the protective effect of constitutive skin pigmentation in Negroes both with regard to erythemal sensitivity and other forms of cellular damage induced by UVR.

PHOTOTUMORIGENIC POTENTIAL OF BROAD-BAND (280-340nm) AND NARROW-BAND (311-313nm) PHOTOTHERAPY SOURCES IN HAIRLESS MOUSE SKIN.

N.K. Gibbs, N.J. Traynor, [1]I. Campbell, [1]R.M. MacKie and B.E. Johnson. Photobiology Unit, Univ. of Dundee and [1]Dept. of Dermatology, Univ. of Glasgow, Scotland.

The new Philips TL01 narrow-band (311-313nm) source and conventional broad-band sources (eg Philips TL12; 280-340nm) are used in psoriasis phototherapy where treatment regimens are based on a pre-determined minimal acute dose. We have compared photo-tumorigenesis of TL01 and TL12 in mouse skin. Groups of 20 albino Skh-1 hr/hr mice were exposed 5 days/week to ~20%, 40% or 70% of an acute minimal oedema dose (MOD) of TL01 or TL12 radiation. The time for 50% of animals to develop a tumour >1mm (T_{50}) was recorded (TABLE). At sacrifice (T_{max}), the tumour yield (mean tumours/mouse) and the percent of histologically malignant lesions (% SCC) in a random sample of 20 tumours/group were scored (TABLE).

% MOD	LAMP	DOSE mJ/cm²	T_{50} Wks	T_{max} Wks	YIELD	% SCC
20	TL01	85	32	42	7	45
	TL12	11	46	50	4	5
40	TL01	184	22	34	19	55
	TL12	27	30	40	11	15
70	TL01	324	20	24	7	5
	TL12	51	24	34	15	15

With 20% and 40% MOD, TL01 tumour incidence ($p<0.0001$), yield and malignancy was greater than TL12. This difference was less marked at 70% MOD ($p<0.018$ for incidence) where TL01 irradiation stopped at 16 wks due to irritation. The possibility of increased tumour risk with TL01 phototherapy should be considered and balanced against the high phototherapeutic efficacy of this source.

A COMPARISON OF THE ERYTHEMAL RESPONSE TO PUVA USING ORAL 8-METHOXYPSORALEN (8-MOP) AND BATH TRIMETHYLPSORALEN (TMP)

L E.Rhodes and P.S.Friedmann, Dept. of Dermatology, Royal Liverpool University Hospital, Liverpool

Patients treated with topical TMP PUVA are observed to burn more than those receiving the oral 8-MOP regimen. We have examined the dose-response relationship in these 2 commonly used regimens to see whether the slope or position of the curves could explain the difference.

26 patients with chronic plaque psoriasis received either oral 8-MOP 0.6mg/kg 2 hours before UVA testing (14 patients) or a 10 minute bath in a 50µg/l TMP-water solution immediately before UVA exposure (12 patients). The 2 groups were of comparable sun-reactive skin type distribution. The back was irradiated with a series of 6 doses of UVA, 0.5-5 J/cm² for bath TMP and 1.5-13.5 J/cm² for oral 8-MOP, using a fluorescent UVA lamp (Waldmann 800). At 72 hours, the sites were examined for visible erythema and the minimal phototoxic dose (MPD) noted. Erythemal readings were taken in triplicate at each site using a reflectance instrument and the increase in erythema over background was plotted against log UV dose. A logit function was fitted to the data to construct sigmoid dose response curves.

The median MPD value for UVA was 0.75 J/cm² following bath TMP and 5 J/cm² after oral 8-MOP ($p<0.001$), a potency difference of 6.7. Analysis of the dose-response curves showed no difference in the slopes of the 2 groups. The calculated mean threshold dose i.e. the UVA dose where the extrapolated linear regression line intersects the x axis, was lower for bath TMP than for oral 8-MOP, 0.42 ± 0.5 J/cm² and 2.36 ± 1.6 J/cm² respectively ($p<0.01$). No relation was found between skin type and MPD, threshold dose or slope of response, but the subgroups are very small

Thus topical TMP at a concentration of 50µg/l, photosensitizes to a greater degree than oral 8-MOP 0.6mg/kg, requiring 6-7 times less UVA. Since the dose-response curves are parallel this indicates firstly that the increased photosensitivity to topical TMP is not a qualitative difference (steeper slope) and secondly, it suggests a common mechanism for production of erythema.

RESPONSE OF PSORIASIS TO PSORALEN UVB PHOTOCHEMOTHERAPY (PUVB).

A Sakuntabhai, PM Farr and *BL Diffey. Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne. *Regional Medical Physics Department, Dryburn Hospital, Durham.

Psoralen photochemotherapy is usually performed using UVA fluorescent lamps with peak emission around 352 nm. An improved response of psoriasis to PUVA has been shown[1] with lamps peaking at 325 nm but the effect of shorter wavelengths has not been determined. Narrow band (311±2 nm) UVB fluorescent lamps (Philips TL01) have been developed for phototherapy of psoriasis; we now report their use in psoralen photochemotherapy.

Eight patients with plaque type psoriasis involving both forearms were studied. In each patient one forearm, chosen at random, was treated twice weekly with 0.7 J/cm^2 from a unit containing TL01 lamps. Immediately after irradiation patients were given 8-methoxypsoralen (0.6 mg/kg) and 2h later the other arm was treated using the same dose of radiation. For both arms equal dose increments were used of up to 40% each week. Response was assessed at weekly intervals by an investigator, unaware of the treatment allocation, who compared the severity of psoriasis on either arm.

One patient failed to attend after one week of treatment; the remaining 7 patients completed the trial. The 8-MOP TL01 treated arm cleared before the TL01 treated arm in 6 patients and clearance was equal in one patient ($p=0.02$, McNemar's test). The median number of treatments required for clearance with 8-MOP TL01 was 6 (range 4-6) with a median cumulative UVB dose of 4.5 J/cm^2 (range 3.7-5.7).

These results show for the first time that psoralen-UVB therapy is effective in psoriasis, and the rapid response of psoriasis in this study suggests that 8-MOP TL01 (PUVB) treatment may be more effective than conventional PUVA which uses lamps emitting longer wavelength radiation.

[1] Farr PM, Diffey BL, Higgins EM, Matthews JNS. The action spectrum between 320 and 400 nm for clearance of psoriasis by psoralen photochemotherapy. *Br J Dermatol* 1991; 124: 443-8.

VARIABLE PHOTOSENSITIVITY AND CLINICAL RESPONSE OF PSORIASIS TO PUVA WITH CONVENTIONAL 8-METHOXYPSORALEN DOSAGE.

A Sakuntabhai, PM Farr and *BL Diffey. Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne, and *Regional Medical Physics Department, Dryburn Hospital, Durham.

In PUVA therapy 8-methoxypsoralen (8-MOP) is conventionally given at a dose of 0.6 mg/kg. The availability of crystalline 8-MOP as 10 mg tablets means that actual doses are calculated to the nearest 10 mg, with patients receiving either 30,40,50 or 60 mg depending on their weight. We have investigated the appropriateness of this dosing system.

Plasma psoralen concentration was measured by HPLC in 41 patients 2 h after ingestion of crystalline 8-MOP (0.6 mg/kg). Patients receiving 30 mg of 8-MOP had significantly lower plasma 8-MOP concentration than patients receiving 40, 50 or 60 mg ($p=0.01$, Kruskal-Wallis test). However, in the same patients when dosed according to body surface area (25 mg/m^2), plasma 8-MOP concentrations were not significantly different between dose groups.

Minimal phototoxic dose (MPD) measurements were made 72 h after irradiation using UVA fluorescent lamps (Philips TL09) in 251 patients given 0.6 mg/kg 8-MOP. Patients receiving 30 mg of 8-MOP had significantly higher MPD values than patients receiving 40, 50 or 60 mg ($p=0.001$, Kruskal-Wallis test). In addition there was a higher proportion of patients in the 30 mg 8-MOP group who did not react to the highest UVA dose (10J/cm^2) given during phototesting ($p=0.008$, Chi-square test)

Clinical response was judged by further analysis of the previously reported[1] study of 100 patients with psoriasis given twice weekly PUVA treatment. Patients receiving 30 mg of 8-MOP required a significantly greater number of exposures for clearance than patients receiving 40, 50 or 60 mg ($p=0.04$, proportional hazards survival analysis).

Calculation of 8-MOP dose according to body weight (0.6 mg/kg) is inappropriate as those 20% of patients who receive 30 mg show reduced photosensitivity and poor clinical response. Calculation of 8-MOP dose according to body surface area rather than body weight reduces variability of plasma 8-MOP concentration and should improve clinical response.

[1] Sakuntabhai A, Sharpe GR, Farr PM. Improved efficacy and efficiency of PUVA in psoriasis with twice weekly treatment. *Br J Dermatol* 1991; 125 (Suppl 38): 28.

CELLS IN THE BULGE OF THE MOUSE TELOGEN FOLLICLE GIVE RISE TO THE LOWER ANAGEN FOLLICLE.

CL Wilson, T-T Sun*, RM Lavker. Dept of Dermatol, Univ of Pennsylvania, Philadelphia, Pa, *Dept of Dermatol and Pharmacol, New York Univ, New York, NY.

The hair follicle undergoes a regular cycle of growth (anagen), involution (catagen) and rest (telogen). With each new cycle, both outer root sheath and matrix are regenerated. We proposed that the relatively primitive, slow-cycling cells in the bulge of the outer root sheath are the hair follicle stem cells (Cell 1990;61:1329). As stem cells, upon division, they would give rise to a population of transient-amplifying cells which would differentiate into outer root sheath and matrix keratinocytes. This contrasts with the belief that the matrix keratinocytes migrate upwards in catagen and later produce the new lower follicle. Bulge cells located at the insertion site of the arrector pili muscle are easily distinguished by their characteristic convoluted nuclei. The lowermost part of the telogen follicle is composed of bulge cells which therefore lie in close proximity to the dermal papilla. Demonstration that the normally quiescent bulge cells proliferate at the onset of anagen would be evidence for their role in the formation of the outer root sheath and matrix.

In the mouse the first hair growth cycle is characterised by rapid follicular neogenesis, but the second and third cycles follow the normal follicular growth pattern We examined the onset of anagen in the third hair cycle as this is slower in onset than the second and has a cranio-caudal wave of hair growth. Two litters of albino (SENCAR) mice (28 animals) were followed from birth, and the stages of the hair growth cycle identified by hair dying combined with shaving. At the end of the second telogen judged by the first sign of white hair (age 68-82 days), animals were given colchicine (10µg, intra-peritoneal), and biopsied after 8 hours. Specimens were taken across the growth wave which included hair in telogen and all phases of anagen. Mitoses were seen in bulge cells during early anagen (phases I and II). The bulge cells were quiescent by anagen III; no mitoses were seen in these cells at any other stage. This confirms that they proliferate transiently, solely at the onset of anagen and strongly supports the suggestion that bulge cells are the origin of the whole lower follicle in anagen.

REGULATION OF GLYCOSAMINOGLYCANS IN THE EXTRACELLULAR MATRIX OF CULTURED HUMAN HAIR PAPILLA CELLS.

M Taylor, ATT Ashcroft, AG Messenger, Department of Dermatology, Royal Hallamshire Hospital, Sheffield, S10 2JF.

During anagen the extracellular matrix (ECM) of the hair follicle dermal papilla (DP) is rich in glycosaminoglycans (GAG) including chondroitin (CO), chondroitin sulphate (CS) and heparan sulphate (HS). Immunostaining for CO and CS, but not HS, is lost as the follicle enters telogen. To elucidate how these changes may be controlled we have studied the effect of a number of cytokines and growth factors on the production of GAG by cultured DP cells.

Both medium and ECM GAG synthesised by sub-cultured confluent human DP cells were determined by radiolabelling with ^{35}S-sulphate and ^{3}H-glucosamine and liquid scintillation counting. Specific enzyme/chemical depolymerisations were performed to identify individual GAG.

Transforming growth factor-β (TGF-β, 2.5ng/ml) stimulated ECM GAG by 45% (+/-12%) increasing CS, dermatan sulphate (DS) and HS to a similar extent but had little effect on medium GAG (n=6 experiments). Interleukin-1 (IL-1β, 2u/ml) and tumour necrosis factor-α (TNFα 50u/ml) stimulated total GAG synthesis into the medium by 35% (+/-10%) but had no effect on total GAG in the ECM (n=9). However, both cytokines reduced CS (50-70%) and DS (25-45%) in the ECM but increased HS (15-60%). Insulin-like growth factor-1 and epidermal growth factor had no effect on GAG synthesis.

These results suggest that TGF-β may upregulate ECM production of GAG in the anagen DP. IL-1β and TNFα reduce the proportion of total GAG which is retained in the ECM and induce changes in GAG species which resemble those that occur in the DP during catagen. Thus, TGF-β, IL-1β and TNFα may be involved in the intrinsic regulation of DP GAG in vivo.

EPITHELIAL KERATIN DISTRIBUTION IN ISOLATED AND EGF TREATED HUMAN HAIR FOLLICLES.

GE Westgate, IM Leigh[*], CM Wilson[**], SM Burge[**], T Kealey[′] and MP Philpott[′] Biosciences Division, Unilever Research, Colworth House Sharnbrook, Bedford, UK [*]Dept of Experimental Dermatology, The London Hospital, London, UK. [**]Dept of Dermatology, Slade Hospital, Oxford, UK. [′]Dept of Clinical Biochemistry, University of Cambridge, Addenbrookas Hospital, Cambridge, UK

The distribution of epithelial keratins in hair follicles in skin has been described previously However, the recent development of a method to isolate and culture human hair follicles has enabled investigation of the distribution of epithelial keratins in response to growth modulating factors *in vitro*. In this study we have examined the changes in epithelial keratin distribution in follicles treated with epidermal growth factor (EGF), known to cause a catagen like morphology after 3-4 days in culture. We used monospecific monoclonal antibodies to the full range of epithelial keratins (K1,10, K5,14, K6,16, K7, K8,18, K19) in immunoperoxidase staining experiments.

Results showed that the pattern of expression in freshly isolated follicles was as previously observed in scalp sections. Very little change was observed over 3 days in control cultures, with cells expressing outer root sheath (ORS) keratins extending further around the hair bulb in comparison with freshly isolated follicles. The most interesting changes were observed following 3 days incubation with 10ng/ml EGF. Cells expressing the ORS keratins 5 and 14 were observed within the bulb matrix, either side of the dermal papilla Induction of the upper ORS keratins 6 and 16 and the inner root sheath keratin 7 was also observed in cells extending into the lower follicle and bulb matrix, contrary to the controls. There was also some expression of simple epithelial keratins 8 and 18 in cells of the innermost layer of the ORS, deep in the bulb Keratin 19 expression has been previously described in cells of the bulge region of the follicle i.e. not within the portion of the follicle isolated. The expression of K19 was variable in the ORS in control cultures and generally increased in the EGF treated follicles. However, it was interesting that cells expressing K19 were not increased in the bulb matrix or in the extended part of the cultured follicles. The epidermal keratins K1 and 10 were not expressed in any cells of the cultured or EGF treated follicles. This change in keratin distribution with EGF is supported by the change in pattern of proliferating cells observed by ^{3}H thymidine labelling after incubation in EGF. This showed rapid proliferation in the ORS and loss of proliferation of bulb matrix cells.

These results indicate EGF causes rapid changes in the fate of the bulb matrix cells from pre-trichocyte to an ORS and not an epidermal type and demonstrate the plurality of differentiation pathways available in the bulb matrix.

CHANGES IN HUMAN HAIR-SPECIFIC KERATIN GENE EXPRESSION DURING THE HAIR CYCLE,

M.B. Hodgins, G. Parker, R. Choudhry, S. Hainey* and P.E. Bowden*, Dept. of Dermatology, University of Glasgow and Dept. of Biological Sciences*, University of Dundee.

Hair-specific keratin intermediate filament (IF) proteins are good molecular markers of hair differentiation. Recently, we described the cloning of a DNA fragment encoding a human type I hair-specific keratin gene (HSK-1). The expression of this gene (HSK-1) has now been studied at different stages of the hair growth cycle and compared to K14, a keratin expressed in epidermal basal cells and outer root sheath (ORS) of the hair follicle. Specific keratin mRNA's were detected in paraffin sections of hair follicle containing human skin by in situ hybridization with HSK-1 and K14 specific ^{35}S-riboprobes prepared from cloned DNA fragments (pGEM-3Z vector). Catagen follicles are rare but as human hair follicles cultured in Eagle's medium enter a catagen-like state after 6 days, this material was also examined. HSK-1 mRNA localised specifically to the precortical cells in anagen hair follicles and was not seen in the medulla, germinative matrix, ORS, inner root sheath, epidermis or other appendages. No expression was observed in telogen follicles or in early anagen prior to complete formation of the new germinative matrix. In contrast, K14 mRNA was restricted to the ORS in anagen follicles but was expressed in the epithelial cells surrounding the club hair root in telogen/early anagen. No K14 expression was observed in new germinative matrix cells in early anagen. HSK-1 expression was seen in precortical cells of cultured hair follicles even when hair growth had ceased and the germinative matrix had degenerated. The transition of these follicles into a catagen-like state was preceded by the appearance of K14 mRNA in cells of the germinative matrix. These results demonstrate that HSK-1 gene expression is specific to hair cortical cells in anagen, reduces in catagen, is absent in telogen and not activated until anagen is completely re-established. K14 gene expression also changes during the hair cycle, being induced in matrix cells during transition from anagen to catagen and prominent in the epithelial cells that surround club hairs in early anagen.

CHARACTERISATION OF HUMAN COSMID CLONES CONTAINING TYPE II HAIR-SPECIFIC KERATIN GENES.

S. Hainey, A.P. Withers and P.E. Bowden, Dept. of Biological Sciences, University of Dundee, UK.

Keratin intermediate filament (IF) proteins are major structural components of the skin and its appendages. Hair-specific keratin genes encode the filament forming proteins of the hair shaft and their expression in hair bulb trichocytes is regulated during the hair growth cycle. Isolation of complete hair-specific keratin genes and their regulatory regions would provide useful clones to study hair differentiation at the molecular level. We now describe the isolation of two 40 Kb human cosmid clones (5-2a & 9-1b) which contain type II hair-specific keratin gene sequences.

A human placental cosmid library was screened with a PCR generated labelled DNA probe (hHaKB-2) to the 3' end (2B helical and C-terminal encoding regions) of a human type II hair-specific keratin. Several positive clones were isolated and purified to homogeneity. Restriction enzyme digests demonstrated that only two related (possibly overlapping) clones had been isolated. Southern blotting of restriction enzyme digested (Bam HI, Cla I, Eco RI, Eco RV, Hind III, Kpn I, Pst I) cosmid DNA with the labelled hHaKB-2 probe, clearly demonstrated the presence of hair-specific keratin genes. Subclones (pGEM-3Z) of the Eco RI fragments (11 Kb, 8.5 Kb, 6 Kb, 4.5 Kb, 2.5 Kb, 1.3 Kb, 1.2 Kb, 0.9 Kb) were then characterized by mapping and sequencing.

Cosmid vector sequences were identified on subclones 9E1 (8.5 Kb vector), 9E3 (2.5 Kb; Cos T7 promoter) and 9E7 (4.5 Kb, Cos T3 promoter). One clone (9E9, 6 Kb) had an internal Eco RI site at the 5'end (590 bp from end) and encoded exons 1-6 of a type II hair keratin gene. Exons 7-9 of a type II hair keratin gene were found on another clone (9E8, 11 Kb). The coding sequences were homologous to a mouse type II hair keratin cDNA (77-88 % at the DNA level and 80-91 % at the amino acid level depending on exon) and the 9E8 sequence was almost identical to the hHaKB-2 probe. Preliminary data suggests these cosmid clones contain a second linked keratin gene as well as regulatory sequences. These clones make excellent tools for the further study of human hair-keratin gene regulation.

QUANTITATIVE EVALUATION OF TOPICAL 5% MINOXIDIL IN THE TREATMENT OF DIFFUSE ANDROGEN-DEPENDENT ALOPECIA (DADA) IN FEMALES

DH Rushton & [1]DA Fenton School of Pharmacy & Biomedical Sciences, University of Portsmouth, Portsmouth, & [1]Dept. of Dermatology, St Thomas Hospital, London.

In men, 2% topical minoxidil has proved disappointing in the treatment of male pattern baldness *(De Groot et. al., Lancet 1986, 1019-21)* Diffuse androgen-dependent alopecia in women (Ludwig pattern) is a genetic condition mediated by androgens and is associated with a conversion of terminal hair to vellus hair, and a reduction in the number of productive hair follicles. In addition, optimal iron stores (serum ferritin >40μg/L) appear critical for hair regrowth *(Rushton & Ramsay, Clin Endocrinol 1992, 36; 421-27)*. The efficacy of topical 5% minoxidil was assessed in 9 women aged between 25-68 years exhibiting DADA, in whom serum ferritin levels were >40μg/L Hair variables were evaluated basally and again after 12 months of therapy employing the unit area trichogram, a technique with a proven sampling error of <5%. Compared to baseline, significant changes in mean values were found after 12 months of treatment (Table)

Values for hair variables obtained from 9 women basally and after 12 months of topical 5% minoxidil therapy. *(Data are presented as Mean ± SD)*

Variable	Basal	After 12 months	Significance
Total hair cm^{-2}	161 ± 40	199 ± 48	†P < 0·001
Non-vellus hair cm^{-2}	130 ± 43	161 ± 55	†P < 0·001
Anagen phase (%)	78·9 ± 8·7	83·1 ± 6·7	*P = 0·06
Vellus hair (%)	20·3 ± 12·9	19·2 ± 12·7	NS

† Student's *t* test (paired samples), * Wilcoxon signed rank test

These findings indicate that in DADA growth inhibited follicles are present and topical 5% minoxidil can initiate some meaningful follicular activity Whether such a response is dose related remains to be established These changes are not observed in men with male pattern baldness (Hamilton patterns); however, in males with a diffuse (Ludwig) pattern topical minoxidil solutions may prove beneficial and warrants further investigation.

PATTERNS OF KERATIN EXPRESSION IN THE NAIL UNIT - AN INDICATOR OF REGIONAL MATRIX DIFFERENTIATION

D.de Berker, I.Leigh† and F.Wojnarowska
Dept. of Dermatology, The Slade Hospital, Oxford and †The Department of Experimental Dermatology, The Royal London Hospital, London

A range of monoclonal antibodies (Mab) to epithelial keratins was used to differentiate regions in the nail unit The findings were considered in the light of theories of nail formation.

Multiple cryostat sections of nail unit were examined from 3 accessory digits removed within the first 2 years of life. Abdominal skin and scalp were used as controls. Thirteen keratin specific Mabs were used, including trichocyte Mabs, LP1k, LP2K and CAM5.2. The conformation specific Mab, LH6, and positive (LH7.2.Collagen VII) and negative (tissue culture medium) controls were also employed. Antibody binding was demonstrated using standard immunoperoxidase techniques

Keratin distribution was recorded according to the anatomical model of Lewis in which Dorsal (DM), intermediate (IM) and ventral (VM) matrix was described. Ventral matrix is synonomous with nail bed (Lewis BL Arch Dermatol.Syphilol 1954,70.732-44)

LH2(K10) and LL017(K1) were demonstrated suprabasally in normal skin, DM and IM and were absent in VM. The trichocyte keratin, CAM5.2(K7/8) is normally associated with the inner root sheath of the hair folllicle and was seen solely in the DM. LP1K(K7), has a similar specificity but although it was absent in normal skin it was present in a patchy distribution in the skin of the proximal nail fold and finger pulp It was seen suprabasally in the DM and IM, but but not in the VM The discrepancy is likely to be due to different sensitivities.

LH6 was seen in the basal and epibasal layers throughout the nail unit apart from the VM, where it was also suprabasal. C46(K7/17), normally only in hair follicle and sweat gland, was also seen throughout the VM, sparsely in the IM and was absent in the DM. Conversely, the hyperproliferative keratin, K16 (LL025), was seen suprabasally in the VM and DM but not at all in the IM.

The expansion of the basal markers in the VM is likely to correspond to the absence of the suprabasal keratins, which might normally mask the basal epitopes. This absence of suprabasal differentiation might be used as an argument in favour of the VM not contributing to nail formation. In summary, keratin distribution can be used to distinguish areas of nail matrix. The pattern of distribution within these structures increases our understanding of how different parts of the matrix contribute to nail formation.

THE BASEMENT MEMBRANE ZONE OF THE NAIL.

Rodney D. Sinclair, Fenella Wojnarowska, Irene M. Leigh* and Rodney P.R. Dawber, Department of Dermatology, Slade Hospital, Oxford, and *Department of Experimental Dermatology, London Hospital Medical College, London.

The anatomy of the epidermis, dermis and subcutaneous tissues of nail apparatus is distinct from that of non-appendageal skin. Apart from the demonstration of the longitudinal configuration of the dermal-epidermal junction of the nail bed there have been no studies of the composition of the basement membrane zone of the nail apparatus. We obtained three human accessory digits, including one thumb, all of which had been amputated for cosmetic reasons and were without known pathology. Specimens were stained with a range of monoclonal and polyclonal antibodies which target normal basement membrane zone antigens and studied by indirect immunofluorescence.

This study demonstrates that the four distinct regions of the nail, namely the proximal nail fold, the nail matrix, the nail bed and the hyponychium, had normal expression of the epidermal associated antigens, the 220 and 180 kDa Bullous Pemphigoid antigens, and the $\alpha 6\ \beta 4$ integrin. Interestingly the dermal associated components, namely the 285 kDa linear IgA antigen, the extracellular matrix glycoproteins chondroitin sulfate, type VII collagen and its closely associated proteins and the poorly characterised antigens for LH 24 and LH 39 were also expressed normally. Fibronectin and Tenascin, which are not normal basement zone components were absent.

These findings show that despite the numerous distinct features of epidermis, dermis and subcutis of the nail apparatus, the basement membrane zone has identical antigen expression to that of the dermal-epidermal junction elsewhere.

EXAMINATION OF COGNITIVE BIASES INHERENT IN THE ASSESSMENT OF DISEASE AREA BY PHYSICIANS.

S Tiling-Grosse, J.L.Rees, Department of Dermatology, University of Newcastle upon Tyne

Most clinical investigators accept that the PASI or allied scoring systems are problematic: the inability to assess disease extent accurately severely limits the statistical power of comparative trials of psoriasis treatments. Despite this, there has been little interest paid to the underlying cognitive difficulties associated with the assessment of disease area. In the present study we have used schematicised figure outlines with areas of shading to assess the ability of a variety of individuals from different backgrounds and different training in assessing area of involvement. 49 dermatologists or laboratory staff or nurses or other physicians were asked to assess area on 30 figure outlines which had been chosen so as to allow comparisons to be made between number of lesions, symmetry, extent of lesions and pattern. Large and statistically significant results ($p<0.05$, Fisher's Exact test on unordered contingency tables) were evident between different groups. Nurses, medical students and physicians tended to overestimate body area considerably, as did most dermatologists. However, there was an interaction between extent of area of involvement and the degree of error. By controlling for the extent of error it was still clear that a large residual variance was due to the pattern of some of the body areas and was independent of the extent of the disease. In addition, we found that physicians who had received additional training in the assessment of disease severity had a far greater consistency than those who had not. This suggests to us that training (perhaps by the use of a number of standardised figure outlines) would improve physicians' ability to assess area of disease involvement and make measurement techniques such as the PASI more sensitive and reproducible.

FILARIAL-SPECIFIC HUMORAL IMMUNE RESPONSES IN CUTANEOUS MESOENDEMIC SAVANNAH ONCHOCERCIASIS IN NORTHERN NIGERIA IgG_4 SUBCLASS SPECIFICITY

M E Murdoch[1,4,5], T Garate[6], R M.E. Parkhouse[6], R M Maizels[6], R.J Hay[4], A Abiose[1,2], B.R. Jones[3]

WHO Project on immunopathology of onchocerciasis ID 890134 based at the Guinness Ophthalmic Unit, Ahmadu bello University[1], the National Eye Centre[2], Kaduna, Nigeria and Institute of Ophthalmology, London[3], and the Institute of Dermatology[4], Imperial College of Science, Technology & Medicine[5] and NIMR, Mill Hill, London[6]

Onchocerciasis, a filarial infection caused by *Onchocerca volvulus*, is endemic throughout tropical Africa. At present no serological test is available and the current method of diagnosis relies upon the demonstration of microfilariae in skin snips. As a preliminary step towards finding a sero-diagnostic tool, a quantitative assessment of filarial-specific humoral immune responses was made in 125 individuals within the context of a WHO/TDR community-based ivermectin trial in mesoendemic onchocercal villages in Kaduna State, Northern Nigeria

Patients were selected for 10ml venous blood samples in the following groups: Acute Papular Onchodermatitis (APOD, n = 13), Chronic Papular Onchodermatitis (CPOD, n = 17), Lichenified Onchodermatitis, (LOD, n = 4), Atrophy (ATR, n = 40), Depigmentation (DPM, n = 19), and Normal Skin, High Microfilarial Load (NSHMF, n = 32) using our previously described clinical classification system (Murdoch ME, Hay RJ, Ramnarain N, Lucas S, Jones B, Abiose A. A clinical classification and grading system of the changes in onchocerciasis and histopathological findings. *Br J Dermatol* 1990,123(37 Suppl).28). Sera from Nigerians living in a rural area non-endemic for onchocerciasis (NONEND, n = 16) and pooled groups of sera from healthy Europeans and Loa-Loa, Mansonella and Wuchereria bancrofti infected individuals served as controls.

Total IgG and IgM immunoglobulin class and IgG_1 IgG_2, IgG_3 and IgG_4 subclass responses were determined by standard ELISA technique using phospate buffered saline (PBS) extract of adult female *O volvulus* worms as antigen at a concentration of 2µg/ml.

The IgG_4 optical density results for each category of onchocercal skin disease were significantly higher than NONEND ($p<0\ 02$ for all clinical subgroups, Wilcoxon rank sum test). This subclass also demonstrated least background reactivity with pooled European and other filarial infection sera Marked differences between the various onchocercal clinical groups were not found, except for LOD which had high total IgG and $IgG_{1,2,3}$ and lower IgG_4 responses

Background reactivity plus the cross-reactivity between nematodes is known to be caused by antibodies to phosphorylcholine and carbohydrate determinants which are subclass restricted in humans and absent in the IgG_4 subclass. The current results therefore confirm the previous report of enhanced specificity of the IgG_4 subclass in onchocerciasis (Weil GJ, Ogunrinade AF, Chandrashekar R, Kale OO. IgG_4 subclass antibody serology for onchocerciasis *J Infect Dis* 1990;161 549-54). This is the first isotype-specific analysis using *O volvulus* antigen with detailed cutaneous clinical correlation.

Acknowledgements This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (ID 870456 and 890134), the Leverhulme Trust, and the Royal Commonwealth Society for the Blind. MEM is supported by the Wellcome Trust

THE EFFECT OF SMOKING ON CUTANEOUS REACTIVITY.

C M Mills & R Marks. Department of Dermatology, University Hospital of Wales, Heath Park, Cardiff, CF4 4XW

Cigarette smoking is a risk factor for subsequent development of psoriasis(1), but appears to confer protection against developing severe acne(2). Smoking has complex pharmacological and immune modulating effects, but the mechanism by which it alters expression of skin disease is unknown.

We have examined the effect of cigarette smoking on cutaneous inflammation and immune function in 25 volunteers smoking more than 20 cigarettes per day and 25 controls matched for age, sex and skin type. The following tests were performed; Intradermal histamine injection, hexyl nicotinate induced erythema, erythema induced by 2x minimal irritant dose (MID) of sodium laryl sulphate (SLS), erythema following 2x minimal erythema dose (MED) of UVB, reactive hyperaemia of forearm skin following arteral occlusion, skin prick testing with common allergens (house dust mite, grass pollen, cat fur and control substance).

No difference was seen in the weal and flare reaction to histamine injection between the two groups, but smokers showed significantly reduced reactivity when challenged with 2x MID of SLS ($p=0\ 003$), and 2x MED UVB ($p=0\ 0001$) measured on visual analogue scale and by erythema meter. There was a decrease in vascular response to topically applied hexyl nicotinate measured by laser doppler flowmetry in smoker ($p<0.05$) and the peak blood flow attained ($p<0.05$). No difference was seen in the resting blood flow between the two groups, or in the reactive hyperaemia following arteral occlusion. Positive skin test reactions in the smokers, 22%, was significantly lower than in controls 65%.

The results show important changes in cutaneous reactivity in smokers, which may help explain the observed effects of smoking on skin disease.

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CUTANEOUS NERVE FIBRE DEPLETION IN VIBRATION WHITE FINGER

P C Goldsmith, C B Bunker, T A Leslie, G Terenghi *, F Abadia Molina* , J Polak * and Pauline M Dowd Dept of Dermatology, University College and Middlesex School of Medicine, * Dept of Histochemistry, The Royal Postgraduate Medical School, Hammersmith Hospital.London

Vibration white finger (VWF) or Raynaud's phenomenon (RP) of occupational origin is the episodic blanching of the fingers in response to cold in those who work with hand held vibrating tools. Unlike RP persistant paresthesiae and pain are common in the hands and arms and those with the disorder are predominantly male. It is a financially compensatable industrial disorder. At present there is no diagnostic test and claims are awarded on clinical grounds alone. Patients with primary RP and RP secondary to systemic sclerosis have been shown to have a deficiency of the powerful vasodilator and sensory neuropeptide, calcitonin gene-related peptide (CGRP) and normal amounts of the vasoconstrictor endothelin-1 (ET-1) in the digital cutaneous microvasculature We therefore investigated the distribution of immunoreactive CGRP and ET-1 in the microvasculature in digital skin of patients with VWF.

Digital cutaneous biopsies were taken from the dorsal aspect of the proximal phalanx in 16 men with VWF, (mean age 52, range 30-72) and in 26 control male manual workers without a history of exposure to vibrating machinery (mean age 43, range 26-62) from matched digital sites. The diagnosis of VWF was made on occupational history, a characteristic history of attacks of RP and paresthesiae with the absence of trophic changes and sclerodactyly. Clinical examination and investigations performed excluded known associated diseases.

The biopsies were fixed in Zamboni's fixative, transferred to phosphate buffered saline, cryostat blocks were prepared and 10 µm sections were cut. Immunostaining was carried out according to the indirect immunoflourescence method with antisera to protein gene product 9.5 (PGP 9 5 is a pan neuronal marker), CGRP and ET-1.

A marked depletion of both PGP 9 5 and CGRP immunoreactive nerve fibres was found in the epidermis and papillary dermis in the digital biopsies from the VWF patients when compared to the control group. The ET-1 staining was the same in both groups. These findings represent a less selective loss of immunoreactive nerve fibres than occured in primary and secondary RP.This different pattern of neuronal loss may reflect the exogenous nature of the aetiology of the condition and is consistent with both a pathogenetic role in the vasodilator / vasoconstrictor imbalance and in the persistent sensory abnormalities characterising this condition They also provide a diagnostic histological test for VWF.

INHIBITION OF THE ENDOTHELIN-1 MEDIATED FLARE IN THE SKIN FOLLOWING APPLICATION OF TOPICAL 0.05% CLOBETASOL PROPIONATE.

P C Goldsmith, C.B.Bunker, T.A.Leslie, H.A.Bull, J.C.Foreman* and Pauline M Dowd. Dept. of Dermatology and Pharmacology*, University College and Middlesex School of Medicine, London .

The mechanism for the production of vasoconstriction within the skin following the topical application of powerful corticosteroids is not understood, though it has been shown *in vitro* that steroids down regulate the activity of endothelial derived relaxing factor nitric oxide (EDRF-NO) synthase in endothelium (Palmer R. *Br J Pharmacol* 1992;**105**: 11) . In the cutaneous microvasculature the presence and synthesis of the powerful vasoconstrictor endothelin-1(ET-1) has recently been demonstrated . It has also been shown that following intradermal (ID) injection of ET-1 the flare that surrounds the central area of pallor is abolished by pre-treatment with topical capsaicin indicating that this flare is neurologically mediated (Bunker CB *Br. J Dermatol.* 1991;**125**:481). We have therefore investigated the effect of topical 0 05% clobetasol propionate on the production of the flare produced by ID ET-1.

Clobetasol propionate 0.05% cream was topically applied under occlusion to the volar aspect of the forearm of adult volunteers (n=4) with normal skin daily for five days. Vehicle base was applied to the contralateral forearm in the same way. In a constant environment chamber at 21 °C baseline cutaneous blood flow was measured by laser Doppler flowmetry (LDF) at two designated sites in the pretreated areas on each forearm. Intradermal injections of 25µl of normal saline or 25 µl of 2.5 µmol ET-1 were made subsequently into each of these sites on both forearms. Two and ten minutes post injection LDF was repeated at each site. Using planimetry the diameter of the pallor and flare size was measured in millimeters at each site and at each time point and the area of the flare calculated.

There was no statistically significant difference in the ET-1 induced pallor in the steroid and vehicle pretreated forearm skin but a significant reduction ($P< 0.025$) in the area and intensity of the flare response in the steroid pretreated forearm skin. These results indicate that topical steroids do not produce vasoconstriction by modulation of the direct vasoconstrictor effect of ET-1 but reduce the neurologically (substance P) mediated flare which it initiates and indicates a dual role of ET-1 in the control of blood flow in normal human skin.

INVESTIGATION OF THE DOSE-RESPONSE CHARACTERISTICS OF INTRADERMAL HISTAMINE AS A MODEL FOR ITCH.

A.J. Carmichael P. Dykes and R. Marks, Dept of Dermatology, University of Wales College of Medicine, Cardiff.

Investigation of pruritus is thwarted by difficulty quantifying both the afferent and efferent loops of the itch pathway. Intradermal histamine may be a useful model to define the stimulus. In this study itch and urticarial response have been quantified in order to assess the suitability of this model and to determine if the urticarial response correlated with itch.

Twenty normal volunteer subjects, 11 female and nine male, mean age 46.2 years (range 21-75 years) were recruited. According to a single blind protocol, placebo and four tenfold dilutions, in ascending strengths, of 0.1 ml of histamine dihydrochloride (0.1 - 100µg/ml) were injected intradermally into one of six randomly selected sites on both forearms. Itch response was recorded using visual analogue scores every minute for 10 minutes, or until the itch stimulus had abated. Total itch score was measured by the area under the curve against time. Weal and flare areas were measured at five minute intervals for 20 minutes.

All 20 subjects completed the investigations. Only one subject developed itch after the placebo injection. Two subjects had no itch response to any concentration of histamine. Seven subjects demonstrated a coherent rank dose-response to concentrations 0.1-10 µg of histamine. The itch response showed marked intersubject variation. In contrast, the urticarial response showed a clear dose-response effect (Table 1). Itch and urticarial responses did not correlate for a given dose of histamine.

Table 1

Histamine conc(µg/ml)		0	0.1	1	10	100
Itch	Median	0	0	238	2080.5	785.5
	Interquart Range	0-0	0-673	0-901	151-3398	0-2724
Flare(mm^2)	Mean	31	421	1200	1917	2992
	SE	21	63	183	182	314

Itch response to intradermal histamine showed wide intersubject variation, limiting its usefulness as a model for itch. The lack of correlation of itch and urticarial response indicate separate pathways are involved in these two effects.

STUDIES OF THE H3 RECEPTOR AGONIST, R α METHYLHISTAMINE IN HUMAN SKIN

Gina M Kavanagh, [1] M W Greaves and C B Archer University of Bristol, Department of Dermatology, Bristol Royal Infirmary, Bristol, and [1]The St John's Institute of Dermatology, St Thomas's Hospital, London, UK

A novel third histamine receptor (the H3 receptor) has been shown to have a widespread distribution in rat and human tissue This receptor mediates inhibition of histamine synthesis and release Whether the H3 receptor exists in human skin is not known In these studies, we have (a) compared the intradermal effects of the selective H3 receptor agonist, R α methythistamine (RAMHA) with those of histamine in human skin, (b) investigated the influence of terfenadine and cimetidine, HI and H2 receptor antagonists respectively, (c) studied the effect of concurrent administration of RAMHA on codeine phosphate - induced responses, codeine phosphate being a recognised histamine liberator, and (d) compared the effect of a selective H3 receptor antagonist, thioperamide, on histamine, RAMHA and codeine phosphate - induced responses

Twenty five normal volunteers received 10 intradermal injections of 50 ul of normal saline containing RAMHA (0 01, 0.1, 1 0, 10 and 100 ug) or histamine (0 01, 0 1, 1 0 and 10 ug), using saline as a control Intradermal injections were repeated ($n = 10$), at 1 week intervals, after the oral administration of terfenadine and cimetidine Codeine phosphate (0 1, 1 0 and 10 ug) was then injected ($n = 8$), either alone or admixed with RAMHA 100ug, using RAMHA as a control. Finally, 8 subjects received codeine phosphate (1 0 and 10 ug), histamine (1ug), and RAMHA (10 and 100 ug) injected either alone or admixed with thioperamide 10-6 M

RAMHA produced dose - related weal and flare responses that were approximately 10 - fold and 5 - fold less respectively than responses to histamine. Flare responses to RAMHA were significantly inhibited by terfenadine ($p < 0 01$), as were flare ($p < 0 005$) and weal ($p < 0 05$) responses to histamine Weal volume responses to RAMHA were reduced after terfenadine, but these differences did not reach statistical significance Cimetidine did not significantly alter either RAMHA- or histamine- induced responses Codeine phosphate - induced responses were not significantly affected by concurrent administration of RAMHA, and thioperamide was not found to influence codeine phosphate, RAMHA or histamine - induced responses

These data demonstrate that the H3 receptor agonist, RAMHA induces vascular responses in human skin However, attenuation of the flare response by terfenadine suggests that RAMHA has partial H1 agonist effects The failure of RAMHA to inhibit codeine phospate - induced histamine release and the lack of effect of thioperamide on vascular responses are observations against the existence of histamine H3 receptor in the skin

SIGNIFICANCE OF DERMIS FOR GRAFTING CULTURED KERATINOCYTES ON FULL THICKNESS WOUNDS

H.A. Navsaria, [1]T. Kangesu, C.R Jones, L. Churchill, [2]S. Manek, [2]C.J Green, I.M Leigh Department of Experimental Dermatology, The Royal London Hospital, London. [1]RAFT Department of Research in Plastic Surgery, Mount Vernon Hospital, Northwood. [2]Section of Surgical Research, Clinical Research Centre, Harrow

Autologous cultured keratinocytes grafted onto full-thickness wounds "take" poorly and any epidermal cover that is produced is unstable. However, a more stable surface has been reported when keratinocytes were applied to a dermal surface (1). We have developed a skin grafting model using polytetrafluoroethylene (PTFE) skin graft chambers in the domestic pig The chambers isolate individual wounds and prevent epithelial migration from the wound edge.

Dermal grafts were prepared by enzymatic separation of the epidermis from split skin to leave a de-epidermalised dermis (DED). Full thickness wounds were initially grafted with autologous DED and subsequently 7 days later with cultured autologous keratinocytes The wounds were serially biopsied over a period of 6 weeks and processed for histology, immunocytochemistry and electronmicroscopy.

Clinically the grafts were seen to mature over the 6 week period In 14/20 wounds, 40-72% of the wound areas (as assessed by image-analysis) acquired epidermal cover at day 14 In 6/20 wounds epidermal cover was 0-27%.

The skin surface was stable at day 21 At this time point electronmicroscopy and immunohistochemistry demonstrated a continuous, well formed basement membrane The epidermis was initially acanthotic but was histologically mature by day 14 Surprisingly the dermal grafts were broken down by day 14 However, a neo-dermis formed beneath all areas with epithelial cover 21 days after grafting the keratinocytes. In this model we have demonstrated the advantage of providing a dermal bed for cultured keratinocytes

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RETINOIC ACID-INDUCED CHANGES IN GLYCOSAMINOGLYCAN SYNTHESIS BY FIBROBLASTS GROWN AS MONOLAYER CULTURES AND WITHIN CONTRACTED COLLAGEN LATTICES.

M. Edward, E.M. White and R.M. MacKie, Dept. of Dermatology, University of Glasgow.

The mode of action of retinoic acid in stimulating the repair of ultraviolet light damaged connective tissue is unclear. Most in vitro studies involving the effect of retinoic acid on matrix synthesis by fibroblasts have been performed on monolayer cultures. Here we have examined the effect of retinoic acid on glycosaminoglycan (GAG) synthesis by fibroblasts grown as monolayers on plastic and within contracted collagen lattices.

Triplicate confluent adult fibroblast monolayer cultures, and fully contracted collagen lattice cultures were incubated for 48hr in the presence of ^{3}H-glucosamine (5μCi/ml), and $^{35}SO_4$ (20μCi/ml), and in the presence or absence of retinoic acid (10^{-6}M). The GAGS were isolated by DEAE ion-exchange chromatography, and the individual GAGS identified by their susceptibility to degradation by specific enzymes and nitrous acid.

^{3}H-glucosamine incorporation into hyaluronate synthesised by fibroblasts grown as monolayers (12.04 ± 1.21 dpm/10^6cells x 10^{-5}) was reduced by 70% when the cells were grown within contracted collagen lattices. Retinoic acid reduced fibroblast hyaluronate synthesis in monolayer cultures by 35%, and by 25% in collagen lattices. ^{3}H-glucosamine incorporation into heparan sulphate (7.36 ± 0.15 dpm/10^6cells x 10^{-4}) and chondroitin sulphates (22.02 ± 0.92 dpm/10^6 cells x 10^{-4}) in monolayer cultures was reduced by 14% and 49% respectively when the cells were cultured in collagen lattices, while sulphate incorporation was increased by 22% in heparans and decreased by 22% in chondroitins. Retinoic acid increased ^{3}H-glucosamine incorporation into heparan and chondroitin sulphates by 50% and 30% respectively in monolayer cultures, but had little effect in lattice cultures. Sulphate incorporation was not affected in either culture.

These results demonstrate that fibroblasts within a 3-dimensional collagenous matrix exhibit altered GAG synthesis compared to monolayer cultures, and that their response to retinoic acid is different and possibly more analagous to in vivo events.

STUDY OF THE TOPICAL USE OF A RETINYL ESTER CONTAINING CREAM IN THE TREATMENT OF EXTRINSIC SKIN AGEING

C. M. Green, J. L. M. Hawk, St. John's Dermatology Centre, St. Thomas' Hospital, London

Use of the topical retinoid all-trans retinoic acid (tretinoin) has been shown to have some effect in reversing extrinsic ageing of the skin However, the majority of subjects studied have also developed annoying skin irritation and erythema.

Retinyl propionate, an ester of retinol, has now been shown in preliminary studies to be much less irritating to the skin but also apparently effective against skin ageing in the mouse and we have therefore studied its efficacy in the treatment of human extrinsic skin ageing (defined clinically by the presence of at least half of the following: fine wrinkling, coarse wrinkling, tactile roughness, telangiectasia, irregular hypo-and hyper-pigmentation, yellowness, comedones and actinic keratoses) in 80 volunteers.

In a double-blind randomised placebo-controlled study subjects received either 0.15% retinyl propionate cream or its base alone, which they applied twice daily to the face and dorsa of both forearms and hands over a 24 week period in the first instance; 75 subjects completed the study, of whom 59 continued until the end of 48 weeks. Clinical assessments of the above-mentioned parameters of skin ageing were performed at weeks 0,4,12,24 and 48 using a linear analogue scale and skin surface replicas of fine wrinkling around the eye were taken at the same time points and analysed by profilometry using a Hommel tester. Results were analysed using a repeated measures analysis of variance model.

No statistically significant differences were apparent between the active and placebo creams for any parameter after 24 weeks, but there was a trend towards improvement with both preparations with time, greater for skin pigmentation and laxity on the face, fine wrinkling on the forearms and actinic keratoses at all sites in the active group. At 48 weeks the trends were similar and there were still no significant differences but actinic keratoses had reduced virtually to zero in the active group at all treated sites. No significant differences were detected between active and placebo by profilometry although there was a trend towards less fine wrinkling in the active group. The cream was well tolerated; only 3 subjects withdrew due to skin irritation.

It may be that the effects of the preparation are at least in part due to long term moisturising properties leading to a trend towards improvement with both active and placebo, although specific retinoid effects are not yet ruled out. Further studies with a more concentrated preparation, in particular its effect against actinic keratoses, are warranted.

CIMETIDINE REDUCES DAPSONE-INDUCED HAEMATOLOGICAL TOXICITY IN DERMATITIS HERPETIFORMIS (DH)

L.E Rhodes[1], M D.Coleman, M D.Tingle, J L Verbov[1], B K.Park & P S.Friedmann[1] Depts of Dermatology[1] & Pharmacology, University of Liverpool, Liverpool.

Haemolysis and methaemoglobinaemia during dapsone therapy are caused by the hydroxylamine derivative of dapsone. Approximately half the dose of dapsone taken undergoes N-hydroxylation while the remainder is converted to monoacetyl dapsone, which is thought to act as a plasma source of dapsone. Cimetidine inhibits hepatic N-hydroxylation of the drug via cytochrome p450 but does not reduce acetylation, and hence our aim was to see if dapsone-induced haematological toxicity would be reduced by concurrent administration of cimetidine.

Ten patients with DH on stable doses of dapsone ranging from 25-350mg daily were studied at six weekly visits. Cimetidine was taken during weeks 3 and 4 at a dose of 400mg tds (6 patients) and 800mg 1 hour before the dapsone dose (4 patients) Trough and peak levels of methaemoglobin relative to haemoglobin were assayed by spectrophotometry, and of plasma dapsone and monoacetyl dapsone by HPLC 12hr urinary dapsone metabolites were measured and haemoglobin levels and reticulocyte counts taken

Daily dapsone dosage and methaemoglobin were linearly correlated, r = 0 9 ± 0 05 Patients taking cimetidine 400mg tds showed a fall in peak methaemoglobin level from 7 65 ± 3 1 % to 5.61 ± 2.1 %, a reduction in urinary dapsone hydroxylamine from 17 63 ± 8.9 % to 13 5 ± 8.7 % ($p < 0.05$) and an increase in trough plasma dapsone level from 0 018 ± 0 007 %dose/ml to 0 025 ± 0 008 %dose/ml (p<0 01) There was no significant change in these levels on cimetidine 800mg od Five of the 6 patients taking cimetidine 400mg tds showed a slight rise in haemoglobin and a reduction in reticulocyte count, but there was no change on cimetidine 800mg od. No patient experienced any deterioration in their DH.

Thus cimetidine 400mg tds reduces methaemoglobinaemia due to dapsone in DH whilst increasing trough plasma dapsone levels Further studies are required to see whether concurrent cimetidine will reduce dapsone-induced haemolysis and/or dapsone requirement

CHARACTERIZATION OF FIBRILLAR STRUCTURES AT THE DERMO-EPIDERMAL JUNCTION IN RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA.

J A McGrath, A.Ishida-Yamamoto, A.O'Grady, [1]L.Sakai, [2]A Jassim, [3]I M Leigh, R.A J.Eady Dept. of Cell Pathlogy, St John's Institute of Dermatology, St Thomas's Hospital, London [1]Shriner's Hospital-Research, Portland, Oregon, U S.A. Depts of [2]Immunology and [3]Experimental Dermatology, The Royal London Hospital, London

Recessive dystrophic epidermolysis bullosa (RDEB) refers to a group of inherited skin disorders characterized by trauma-induced blistering and scarring. Ultrastructurally, blisters are formed beneath the lamina densa and anchoring fibrils are undetectable or qualitatively or quantitatively abnormal In many cases of RDEB, only thin wisp-like structures of uncertain composition are seen below the lamina densa in both intact and blistered skin To determine the nature of these fine, fibrillar structures, we have examined the dermo-epidermal junction in 11 cases of generalized RDEB, six cases of localized RDEB, and eight normal controls by pre-embedding immunogold electron microscopy using two antibodies to different anchoring fibril components (LH 7·2 and GDA-J/F3), and an antibody to fibrillin, the main protein in basement membrane zone structures known as dermal microfibril bundles. In the RDEB samples, a variety of thin sub-lamina densa fibrillar structures, 3-20nm in diameter, had specific immunolabelling with the anchoring fibril-associated antibodies, despite having only little or no resemblance to normal anchoring fibrils All 17 cases of RDEB expressed some recognizable anchoring fibril epitopes, even though the labelling was quite sparse in most samples from the severest cases The patterns of LH 7·2 and GDA-J/F3 immunolabelling were similar in both intact and separated RDEB skin In intact RDEB skin, the anti-fibrillin immunostaining resembled normal controls with labelling on lattice-like fibrillar structures extending from the lamina densa to the upper and mid-dermis. In areas of blistering, many of the wisp-like 'fibrils' below the lamina densa had definite anti-fibrillin labelling. These wispy structures were morphologically similar to some anchoring fibril antibody-labelled fibrils This study shows that many of the ill-defined fibrils below the basement membrane in RDEB are related to at least two distinct fibrillar structures, anchoring fibrils and dermal microfibril bundles. Fibrillin is expressed normally in unblistered RDEB skin, but in areas of blistering, dermal microfibril bundles are fragmented as a secondary phenomenon. The variability in LH 7:2 and GDA-J/F3 immunolabelling is compatible with an array of primary structural anchoring fibril abnormalities reflecting the diverse clinical spectrum of RDEB

PLASMINOGEN, ADHESION MOLECULES AND RELATED PROTEINS IN CANTHARIDIN INDUCED ACANTHOLYSIS

JA Yell, SM Burge, D Dean. Dept Dermatology, The Slade Hospital, Oxford

Acantholysis is a feature of diseases such as Darier's disease and Hailey-Hailey disease. Immunocytochemical studies have shown internalisation of desmosomal components after acantholysis but the pathogenesis of acantholysis is unclear. Cantharidin induces blistering with suprabasal keratinocyte acantholysis, possibly by protease activation.[1] Plasmin has been implicated in the pathogenesis of acantholysis in Darier's disease and Hailey-Hailey disease. We examined the distribution of desmosomal components in Cantharidin blisters, to compare them with those previously found in Darier's disease and Hailey-Hailey disease.

Two drops of Cantharidin collodion, was applied to the skin of five normal volunteers. A 4 mm punch biopsy of the blister was taken and snap frozen.

Sections were stained with antibodies to plasminogen, desmosomal proteins (dp) 1/2, dp 3, desmosomal glycoproteins (dg) 1, 2/3 and extracellular carbohydrate residues, using the lectins, peanut agglutinin (PNA) and soybean agglutinen (SBA).

Plasminogen was expressed diffusely by acantholytic cells. Acantholytic cells were stained diffusely with dp1/2; there was some loss of linear peripheral fluorescence for dp3, dg1, dg 2/3, PNA and SBA. There was no clumping of stain

These results are similar to those previously obtained in Darier's disease but different from the staining obtained in Hailey-Hailey disease[2]. Extracellular glycosylated portions of adhesion molecules may be lost after acantholysis, perhaps a result of conformational changes, internalisation of extracellular domains or proteolysis Our work has shown increased expression of plasminogen in acantholytic cells in Cantharidin induced blisters but as in Darier's disease and Hailey-Hailey disease, this is probably secondary to acantholysis and changes in the shape of cells.

We conclude that Cantharidin blisters may be a useful model for the study of acantholysis in Darier's disease.

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HETEROGENEITY OF THE LINEAR IGA TARGET ANTIGENS.

F Wojnarowska, PM Collier, J Allen, [1]P Whitehead, [1]IM Leigh Department of Dermatology, The Slade Hospital, Oxford, [1]ICRF Skin Tumour Laboratory, London.

We have previously observed differences in binding patterns of sera of patients with linear IgA disease (LAD) using suction blisters and salt split skin as substrate for the detection of basement membrane zone (BMZ).antibodies. Cylindroma tumours produce an abundant BMZ, and thus seem an ideal substrate for the detection and differentiation of BMZ antibodies.

59 children and adults with LAD were studied. The sera were tested on normal human skin, salt split skin, a cylindroma tumour and a primary culture of cylindroma cells, using a standard immunofluorescence technique. The staining patterns were observed and compared with bullous pemphigoid (BP), epidermolysis bullosa acquisita (EBA) sera and normal human sera.

19 patients were positive on intact skin, this number increased to 30 when split skin was used. 24 patients showed epidermal binding. 6 patients had BMZ antibodies that bound to the dermis. 28 patients were negative, and in one the serum bound only to intact skin.

The cylindroma demonstrated in some cases expression of the LAD antigens around tumour cells and occasionally thick staining around individual cells. The BMZ of the overlying epidermis was often also positive. This pattern was seen only with sera that bound to the epidermal aspect of split skin (10/24) or were negative (14/28). The dermal binding sera bound only to the BMZ of the overlying epidermis (4/6) and not to the tumour.

The cultured cylindroma cells demonstrated cytoplasmic expression of the LAD antigen, only when epidermal binding (15/24) or negative (17/28) sera were used. The dermal binding sera did not bind to the cultured cylindroma cells.

The staining patterns of the epidermal binding and negative LAD sera were indistinguishable from BP sera on the cylindroma cells. EBA sera bound to the cylindroma tumour and the cells, in contrast to the negative findings with the dermal binding LAD sera.

These results suggest that the LAD antigens are heterogeneous.and that those sera that bind to the epidermal aspect of salt split skin are binding a different target antigen from those that bind to the dermis. In addition those sera that are negative on split normal skin are probably directed, in many cases at the epidermal target antigen. Moreover the dermal antigen is clearly different from collagen type VII.

This study was supported by the Skin Disease Research fund.

α-MELANOCYTE-STIMULATING HORMONE RECEPTOR EXPRESSION AND ITS MODULATION ON NORMAL HUMAN MELANOCYTES.

P.D. Donatien, G. Hunt and A.J. Thody, Department of Dermatology, University of Newcastle upon Tyne, UK.

There are reports that α-melanocyte-stimulating hormone (α-MSH) has no effect on melanogenesis in cultured human melanocytes. However, we have shown that α-MSH can affect melanocyte morphology and increase their attachment to extra-cellular matrix proteins. Thus our findings imply that human melanocytes express functional α-MSH receptors and this possibility has been investigated in the present study.

Human melanocytes were grown to subconfluency in MCDB153 in the absence of synthetic mitogens. Cells were incubated for 2h at room temperature with varying concentrations of ^{125}I labelled Nle^{4}DPhe^7-MSH ± 1000X excess cold peptide. Binding was saturable and specific with a K_d value of 4.9 X 10^{-11}M, which is comparable to that for B16F1 melanoma cells (8.3 X 10 ^{11}M). However, compared to B16F1 cells, the level of binding to human melanocytes was low, reflecting the reduced number of binding sites/cells on the latter. After 2 day culture in the presence of 1mM dibutyryl cAMP there was a 62% increase in specifically bound peptide. 10^{-9}M cholera toxin, which activates cAMP-dependent mechanisms, caused a 25% increase in binding while 25nM TPA, one of the actions of which is to activate the protein kinase C system, caused a 24% decrease in binding. Our findings demonstrate that human melanocytes in culture express MSH receptors and that this expression can be modulated by known melanocyte mitogens.

CROSSTALK BETWEEN EPIDERMAL GROWTH FACTOR (EGF) AND INTERLEUKIN-6 (IL-6) DEPENDENT PATHWAYS FOR REGULATION OF KERATINOCYTE PROLIFERATION. V. Kumar, [1]S. Bustin, [1]R. Barnard, A. Dalley, D. Bryan, [2]K. Brown, I. McKay, [1]N. Williams and I. Leigh, Dept. of Experimental Dermatology and [1]Surgical Unit, London Hospital Medical College, [2]Dept of Biochemistry, AFRC, Cambridge.

The aim of this study was to investigate different pathways through which keratinocytes can be activated in response to growth factors. The SVK14 cell line was treated with 100ng/ml EGF in the presence of cycloheximide for 3 hours when mRNA was extracted and analysed by the northern blotting procedure for induction of early response genes A probe for β-actin was used as control. EGF treatment induced a two-fold increase in transcription of hcMG-1 - the human equivalent of an early response gene induced in a rat intestinal epithelial cell line in response to EGF (Gomperts M, Pascall JC, Brown KD. Oncogene 1990, 5 1081-1083). Although transcripts from the IL-6 gene were undetectable in untreated SVK14, they were readily detectable in EGF treated cells, implicating IL-6 as an early response gene in keratinocytes Transcription of the IL-6 gene is under complex control but its induction can be stimulated by NF-IL6, a nuclear factor of the c/EBP family (Akira S, Isshiki H, Sugita T et al. 1990; 9: 1897-1906). NF-IL6 was present at high levels in EGF treated SVK14 cells suggesting that it may act as an intermediary in the induction of IL-6 transcription. Both Transforming Growth Factor α (which works through the EGF receptor) and IL-6 are over-expressed in psoriatic keratinocytes and have been implicated in their excessive proliferation. We have shown that there is crosstalk between the pathways through which these factors activate keratinocytes.

CIRCULATING CYTOKINES AND SOLUBLE ADHESION MOLECULES IN WIDESPREAD INFLAMMATORY SKIN DISEASE. RW Groves, JNWN Barker, *DO Haskard, #C Bird, DM MacDonald. Department of Dermatology, Guy's Hospital, London SE1 9RT, *Department of Rheumatology, RPMS, London, W12 0NN, and #NIBSC, South Mimms, Hertfordshire, EN6 3QG.

Erythroderma is a life threatening state associated with a variety of inflammatory dermatoses. As cytokine induced adhesion molecule expression by vascular endothelium is likely to be fundamental in control of cutaneous inflammation, we have sought to determine plasma adhesion molecule and cytokine concentrations in 10 patients with erythrodermic psoriasis or eczema

Circulating (c) ICAM-1, ELAM-1 and VCAM-1 were determined using an ELISA method. based on standard high reading plasma samples. Cytokine assays were as follows: interleukin-1 (IL-1) by EL-4/NOB-1 bioassay and IRMA, IL-6 by B9 bioassay; IL-3, IL-5 and GM-CSF by TF-1 bioassay; tumour necrosis factor alpha (TNF-α) by ELISA cICAM-1, cVCAM-1 and cELAM were raised in erythrodermics· cICAM-1 patients 50.6 ± 9 6 U/ml (mean ± SEM), healthy controls (n=74) 30 ± 4 U/ml; cVCAM-1 patients 16.1 ± 2.6 U/ml, controls 5 6 ± 0.4 U/ml, cELAM-1 patients 419 ± 81 U/ml, controls 59 ± 11. Although no IL-1, IL-3, IL-5 or GM-CSF were detectable in patients plasma, IL-6 was highly elevated (patients 78 ± 37 U/ml, controls (n=10) 0 U/ml) and increased levels of TNF-α were present in 3/10 erythrodermics.

IL-6 and TNF-α are likely to be important in the systemic disturbance associated with erythroderma, and interference with their effects may be of therapeutic benefit Increased soluble adhesion molecules probably reflects generalised activation of cutaneous endothelium. Monitoring of these parameters in erythrodermic patients may be of future prognostic value

THE INDUCTION PATHWAY OF CYTOKINE GENE EXPRESSION ACROSS THE ADVANCING EDGE OF PSORIATIC PLAQUES. MJ Cork, JB Mee*, J Tarlow*, FS di Giovine*, AIF Blakemore*, C Day+, K Elliott, JHJ Senior, DW Cotton+, SS Bleehen, DJ Gawkrodger, AG Messenger, GW Duff*. Department of Dermatology, *Section of Molecular Medicine and +Department of Pathology, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF.

Numerous abnormalities of cytokine production have been demonstrated in psoriasis, but the identity of the initiating event(s) is not clear. Investigations should not be confined to the cytokine itself, because the biological response is a function of the balance between the production of cytokine protein, the expression of specific receptors and the production of inhibitors.

This study provides a new approach to investigate the initiating and subsequent sequence of events that produce psoriatic lesions. Across the psoriatic plaque active edge/non-lesional interface six 3 mm punch biopsies are taken. Quantitative profiles for cytokines are established using reverse transcription with polymerase chain reaction amplification to quantify cytokine mRNA (initially IL-1 alpha, IL-1 beta, IL-1 receptor antagonist, IL-1 receptor type 1 , IL-8, IL-6, IFN-y and IL-2). In addition cytokine protein, cell phenotype and kinetic status are being detected with monoclonal antibodies.

We have established a gradient of IL-1 alpha mRNA and protein from high levels in normal skin, lower in non-lesional psoriatic skin and decreasing towards the psoriatic plaque, (N = number of experiments = 5).

In contrast the levels of IL-1 beta mRNA are low in non-lesional skin and increase towards the edge of psoriatic plaque (N = 2). The levels of mRNA for the IL-1 receptor antagonist are reduced within the psoriatic plaque (N = 3), but mRNA for the IL-1 type 1 receptor and IL-8 increases into the psoriatic lesion (N = 1). Our results suggest that although the levels of IL-1 alpha are decreased, the biological activity is increased, so permitting the production of other cytokines such as IL-8.

BIOLOGICALLY ACTIVE AND IMMUNOREACTIVE TUMOUR NECROSIS FACTOR ALPHA (TNF-α) IN PSORIASIS.

P. Ettehadi and R.D.R. Camp, St. John's Institute of Dermatology, London.

Locally-produced TNF has previously been proposed to play a role in the pathogenesis of psoriatic lesions, although evidence for the presence of biologically active material is lacking. We have therefore analysed epidermal samples from psoriatic lesions and control skin by using a TNF-α ELISA, a TNF cytotoxicity bioassay incorporating WEHI-164 Cl13/2 cells, and an ELISA for TNF receptor (both p55 and p75). Lesional and clinically normal heel stratum corneum (SC) was homogenised in RPMI 1640 containing 10% foetal calf serum (25 mg SC per 0.8 ml) and supernatants passed through 0.45 um filters. Bioassays were carried out on these filtrates on the same day.

Dilution-related biological activity was found in lesional (289±85 pg/ml, n=9) but not normal (n=4) heel SC extracts. TNF-α ELISA of SC samples revealed higher lesional than control immunoreactivity, measured lesional levels being approximately twice as high as found with the bioassay (529±81 pg/ml, n=12 and 71±16 pg/ml, n=7 for lesional and control samples respectively). ELISA for p55 showed 842+188 pg/ml (n=10) in lesional SC versus 195±135 pg/ml (n=6) in controls. p75 immunoreactivity was lower in lesional SC extracts (311±109 pg/ml, n=10) and undetectable in controls (n=6). Assay of lesional SC supernatants which had been filtered as described, then stored at -20 degrees C prior to thawing and the same WEHI bioassay, revealed little biological activity, possibly due to interference by precipitate (n=7).

Lesional psoriatic SC extracts therefore contain TNF biological activity which we propose is produced in the deeper viable layers and may play a role in inflammatory events including leucocyte adhesion and recruitment. Higher levels were measured by ELISA, the difference either being methodological or possibly due to partial neutralisation of biological activity by soluble TNF receptor, particularly p55. However, receptor levels in the extracts appear insufficient to abolish biological activity. Care is also required in the interpretation of the results of TNF bioassay carried out on biological material which has been stored frozen.

UPREGULATION OF INTERLEUKIN 1α, ITS RECEPTOR, AND INTERLEUKIN 1 RECEPTOR ANTAGONIST LEVELS IN INDUCED LESIONS OF CHRONIC ACTINIC DERMATITIS.

H. du P. Menagé, M. Kristensen,[1] C.Q. Chu,[2] F.M. Brennan,[1] J.L.M. Hawk and S.M. Breathnach, St. John's Institute of Dermatology, [1]Charing Cross Sunley Research Centre, and [2]Kennedy Institute of Rheumatology, London.

Chronic actinic dermatitis (CAD) is a photodermatosis with histological features resembling those of allergic contact dermatitis. It has been proposed that the cytokine interleukin 1 (IL-1) plays an important role in mediating inflammatory processes in the skin. We therefore sought to determine changes in the expression and distribution of IL-1α, its receptor (IL-1R), and the newly described inhibitor of IL-1, termed IL-1 receptor antagonist (IL-1 RA), during the evolution of induced lesions of CAD. Six patients with CAD received 1.5 minimal response doses of solar simulated radiation (Kratos 2500 W filtered Xenon arc solar simulator) to one forearm. Sequential biopsies of irradiated sites were taken at varying intervals of 5 hrs, 24 hr, 48 hr, 72 hr and 6 days after irradiation; a control biopsy from the non-irradiated contralateral forearm was also taken. Histological examination in each case confirmed induction of acute eczematous changes with epidermal spongiosis at 48 hr. Immunohistochemical staining was carried out on cryostat sections of the skin biopsies, using the APAAP technique and specific monoclonal antibodies.

In unirradiated control skin, weak epidermal IL-1α staining was seen in all cases, and was present in a perivascular distribution in 2 of 6 cases. Epidermal and perivascular IL-1α expression was upregulated following irradiation in all 6 cases. This upregulation was seen at 5 hr, peaked at between 24 hr and 48 hr, and appeared to be waning by 6 days. Similar upregulation of IL-R expression following irradiation was seen at 24 hr and 48 hr within the epidermis and around blood vessels; upregulation persisted at 6 days. Of particular interest was the finding that epidermal IL-1 RA expression was also elevated at 24 hr, 48 hr, and 6 days after irradiation. There was in addition marked upregulation of IL-1 RA expression in a perivascular distribution, as well as in association with dermal inflammatory cells, seen at 5 hr and 48 hr and persisting until 6 days. These findings support a proposed pro-inflammatory role for IL-1 in CAD, mediated via its receptor. We also document for the first time upregulation of the naturally occurring IL-1 inhibitor IL-1 RA in inflamed skin, providing *in vivo* evidence in favour of a regulatory role for this protein in cytokine-mediated inflammation.

Posters

THE IMMUNOREACTIVITY OF NEUROPEPTIDES AND GENERAL NEURONAL MARKER PGP 9.5 IN ATOPIC DERMATITIS. MSK Al'Abadie, HJ Senior, SS Bleehen, DJ Gawkrodger. University Department of Dermatology, Royal Hallamshire Hospital, Sheffield, S10 2JF.

Atopic dermatitis (AD) is an inflammatory skin disorder of multifactorial etiology in which exacerbations may be provoked by either immunological or non-immunological factors. Neurogenic influences are also thought to play a role in the pathogenesis of the disease. In this study we examined the distribution of nerves and neuropeptides in the skin of patients with AD. Immunohistochemical methods were applied using antibodies to general neuronal marker PGP 9.5, substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and neuropeptide Y (NPY). The immunoreactivity and distribution of the neuronal marker and neuropeptides were studied in skin biopsies taken from lesional AD skin (9 patients: 5 males, 4 females), non-lesional AD skin (3 patients), and from normal non-atopic controls (7 subjects).

A semi-quantitative evaluation of the immunoreactivity was made, looking at the epidermis, dermo-epidermal junction, papillary and reticular dermis, and around blood vessels, sweat glands and hair follicles. PGP 9.5 reactivity was increased in the lesional AD skin, mainly at the epidermis and papillary dermis and to a lesser extent around blood vessels (in 6 of 9 cases). SP immunoreactivity was increased only at the papillary dermis of lesional AD biopsies (in 3 of 9 cases). VIP reactivity also showed an increase though this was restricted to the area around eccrine sweat glands (in the same 3 AD lesional biopsies as for SP). CGRP reactivity was increased in the AD lesional papillary dermis (in 4 of 9 cases). NYP reactivity was normal in all patients. The non-lesional and non-atopic control biopsies showed normal reactivity for all antibodies. The findings of this study support the concept of a neuropathogenic factor in the pathogenesis of AD.

A COMPARISON OF THE LOCALIZATION OF THE ANTIBODIES AND ANTIGENS IN CICATRICIAL PEMPHIGOID J. Allen, K Schomberg, V Venning, M. Nayar, and F Wojnarowska, Department of Dermatology, The Slade Hospital, Oxford

The target antigen(s) in cicatricial pemphigoid (CP) remain controversial It has been postulated that it is the bullous pemphigoid (BP) antigen in an extracellular location The site of deposition of the antibodies *in vivo* was compared with the binding site of the antibodies in normal human skin Incubation in 1M NaCl was used to split both the patients' biopsies and the normal human skin The class of immunoreactant was investigated by a standard immunofluorescence technique. Biopsies were obtained from 23 CP patients at initial diagnosis, from skin, oral mucosa and conjunctiva and were re-examined $1^{1}/_{2}$ – 6 years later Sera from 34 CP patients was analysed using split skin to determine the site of binding

Direct	% Biopsies Positive			
	G	A	M	C3
Initial diagnosis	27	0	9	27
1M NaCl stored	36	5	0	9
Epidermal	23	5	0	5
Dermal	5	0	0	0
Epi & dermal	9	0	0	5

Indirect	% Sera Positive			
	G	A	M	C3
Initial diagnosis	29	0	0	6
1M NaCl	39	19	3	13
Epidermal	36	19	3	13
Dermal	3	0	0	0
Epi & dermal	0	0	0	0

The results are shown in the table The circulating antibodies and C3, with a single exception (target antigen 220 kDa), bound to the epidermis The *in vivo* bound antibodies and C3 showed epidermal and combined patterns, and in a single patient dermal binding The pattern was the same for all immunoreactants

There is discordance between the findings in the patients' skin and the binding site of their circulating antibodies This suggests that in CP in some cases, the antibodies are deposited at a dermal extracellular site, differing from the usual site of antigen expression

This work was supported by an Oxford Regional Health Authority Grant

SELECTIN LIGANDS ARE EXPRESSED BY CUTANEOUS SQUAMOUS CELL CARCINOMA (SCC) MH Allen, RW Groves, JNWN Barker, EL Ross, DM MacDonald. Laboratory of Applied Dermatopathology, St John's Institute of Dermatology, UMDS, (Guy's Campus), London

The development of metastasis involves adhesion of tumour cells to vascular endothelium during initial vascular invasion and in deposition of tumour cells at distant sites. Evidence suggests that binding involving endothelial E-selectin and tumour cell expression of its carbohydrate ligands including sialylated- (SLex) and non-sialylated-Lewis-X (CD15) and sialylated Lewis A (SLea) is important in this process.

To investigate the role of such mechanisms in cutaneous tumours, whole skin biopsies were examined immunohistochemically using specific monoclonal antibodies, in SCC (n = 6) expression of both SLex (n = 5) and CD15 (n = 4) was observed focally on neoplastic cells, while SLea was not expressed In contrast, these E-selectin ligands were absent on keratinocytes in both BCC (n = 6) and normal skin (n = 6). E-selectin was strongly expressed on dermal endothelium in SCC and BCC. Three distinct keratinocyte cell lines (A431, HaCat, SVK14) were investigated immunocytochemically and by FACS analysis SLex and SLea were expressed by all three lines, whereas CD15 was only present on SVK14 cells.

Dermal vessels in cutaneous tumours express E-selectin, and SCC but not BCC tumour cells express its cognate ligands These results may explain the lower metastatic potential of BCC and suggest a role for this pathway in early and late metastatic events. E-selectin/carbohydrate interactions may provide a future therapeutic target for anti-metastatic chemotherapy

IMMUNOPHENOTYPING STUDIES IN PITYRIASIS LICHENOIDES

A Anstey , R Thomas, B Jasani, R Marks

Departments of Dermatology and Immunopathology*, University Hospital of Wales, Cardiff CF4 4XW

The aetiopathogenesis of pityriasis lichenoides is uncertain but if the origin of the inflammatory cells was better understood the nature of the disease process involved would be clearer. The aim of this study was to characterise the immunophenotypic features of inflammatory cells involved in pityriasis lichenoides Paraffin-embedded archival specimens from 30 cases of pityriasis lichenoides fulfilling standardised criteria were studied by immunocytochemistry with a panel of seven antibodies. These comprised Factor XIIIa, anti-CD3, UCHL1, KP1, HLA-DR, S-100 and Ki-1(CD30) A two-stage indirect immunoperoxidase method was used

All specimens were negative with Ki-1 antibody. Labelling with Factor XIIIa revealed a mild increase in dermal dendritic cells at the margins of the dermal cellular infiltrate in 10 cases (33%) The activation marker HLA-DR was positive in endothelial cells in 17 cases (56%) and showed patchy positivity in keratinocytes in 7 cases (23%) CD3 positivity demonstrated T-cells as the dominant dermal inflammatory cell ranging from 55 to 80% of cells Most cases showed strong labelling with UCHL1 with the percentage of positive cells within the dermal infiltrate ranging from 43 to 78% Mononuclear phagocytic cells were present in much smaller numbers in all cases as characterised by the KP1 antibody Langerhan's cells were increased in number in association with the focal inflammatory infiltrate in only two cases, as demonstrated by the S-100 antibody

This immunophenotyping study has revealed that in most cases neither epidermal nor dermal dendritic cells are increased in number in association with the focal infiltrate which characterises pityriasis lichenoides. Absence of CD30 expression in all cases does not support the concept of an overlap of pityriasis lichenoides with cutaneous T-cell lymphoma Overall the profile suggests persisting antigenic stimulation from an as yet undetermined antigen.

THE EFFECTS OF TRANSFECTION WITH E6 AND E7 OF HUMAN PAPILLOMAVIRUS-16 (HPV16) ON THE SECRETION OF INTERLEUKIN-6 BY HUMAN KERATINOCYTES.

D. Bryan, [1]C. Sexton, C. Proby, A. Dalley, I. McKay, and I. Leigh. Dept of Experimental Dermatology, London Hospital Medical College, London, and [1]Wellcome Research Laboratories, Beckenham.

We have previously shown that immortalisation of oral keratinocytes with the entire HPV16 genome causes secretion of elevated levels of Interleukin-6 (IL-6) in the derived cell lines. The aim of this study was to elucidate the mechanism of IL-6 induction in HPV immortalised cells. To this end we have chosen two spontaneously immortalised keratinocyte lines HaCaT and LSDM3, which do not harbour detectable HPV genomes The basal level of IL-6 secretion for each of these cell lines has been determined·

	LSDM3	HaCaT
HPV16	Neg.	Neg
IL-6 secretion /10^6 cells	4U/ml (n=3)	20U/ml (n=3)

Individual genes from HPV16, in particular E6 and E7 genes, subcloned into the PJ4Ω expression vector, have been transfected into LSDM3 and HaCaT and clonal cell lines have been derived. The effects of these gene products on levels of IL-6 secretion have been determined. The data support the hypothesis that IL-6 expression is up-regulated by the HPV E6 and E7 gene products, possibly through interaction with the tumour suppressor genes p53 and RB, which are known to down-regulate IL-6 transcription in the HPV - positive HeLa cell line.

PHORBOL MYRISTATE ACETATE ATTENUATES EXPRESSION OF THE pp59^{fyn} CYTOPLASMIC PROTEIN TYROSINE KINASE IN HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS.

Helen A Bull, *P.M. Brickell, Pauline M Dowd. Department of Dermatology and *Medical Molecular Biology, UCMSM, The Middlesex Hospital, London. UK

Mechanisms regulating cell differentiation are poorly understood. However, there is increasing evidence that cytoplasmic protein tyrosine kinases of the c-*src* family are involved in these responses. Recently screening of a human umbilical vein endothelial cell cDNA library identified the *fyn* gene, a member of the c-*src* family which encodes a 59 amino acid peptide pp59^{fyn} with protein tyrosine kinase activity. We have now sought evidence for the expression of pp59^{fyn} in human dermal microvascular endothelial cells (HDMEC) and its potential role in endothelial cell differentiation.

HDMEC cultured from neonatal foreskin have been used for these experiments. Confluent monolayers of HDMEC were incubated with 0.5 M phorbol myristate acetate (PMA) for up to 18 hours. Protein extracts were prepared, size fractionated on SDS-PAGE, electroblotted onto nitrocellulóse and probed with antibodies directed against pp59^{fyn} and phosphotyrosine. The cytoplasmic protein tyrosine kinase pp59^{fyn} was expressed in unstimulated HDMEC and a 59kD band was also detected by the phosphotyrosine antibody. Incubation of HDMEC with 0.5 μM PMA induced rapid attenuation of pp59^{fyn} expression which was evident following 30 minutes exposure and continued up to 18 hours exposure. Incubation for 18 hours with PMA induced a marked change in the shape of the HDMEC from round to spindle shaped cells.

These data demonstrate that HDMEC express the cytoplasmic protein tyrosine kinase pp59^{fyn} and that differentiation of HDMEC induced by PMA is associated with attenuation of pp59^{fyn} expression. Cytoplasmic phosphotyrosine kinases may therefore mediate vascular endothelial cell responses at sites of tissue injury and repair in wounds and in inflammatory dermatoses.

IMPAIRED CUTANEOUS MICROVASCULAR PERFUSION IN PATIENTS WITH CHRONIC VENOUS INSUFFICIENCY (CVI).

R.H. Bull, O. Luetolf, P.S. Mortimer, Department of Dermatology, St. George's Hospital, London SW17, U.K.

Venous hypertension causes leg ulceration by an unknown mechanism. Microcirculatory changes were assessed in the skin of the lower leg of patients at risk of venous ulceration using intra-vital video-capillaroscopy.

The dermal capillaries were examined with the leg supine and dependent in 13 subjects with CVI (7 female, 6 male; mean age 60 8 ± 11.1 years) and in 13 normal controls (6 female, 7 male; mean age 51.7 ± 15.1 years) under native light and during fluorescence angiography (FA).

	CVI		NORMAL	
	SUPINE	DEPENDENT	SUPINE	DEPENDENT
NATIVE	13.1 ± 7.4	12.9 ± 6.9	28.5 ± 4.9	26.8 ± 5.0
FA	15.4 ± 8.1	15.9 ± 8.2	30.7 ± 5.8	28.9 ± 5.3

Table 1. Capillary density (mean ± s.d. /mm^2).

Capillary density was significantly lower in the subjects with CVI compared to the normal controls both in the supine and dependent positions ($p<0.01$). During fluorescence angiography in normal controls fewer capillaries were filled during dependency compared to the supine position ($p<0.01$) but there was no reduction in the CVI subjects.

Fluorescence angiography only highlights perfused capillaries; these results indicate that capillary underperfusion in the gaiter skin of subjects with CVI may be important in the pathogenesis of leg ulceration.

A COMPARISON OF CELL ADHESION MOLECULE EXPRESSION IN LEUKOCYTOCLASTIC AND LYMPHOCYTIC VASCULITIS.

NP Burrows, F Abadia Molina, G Terenghi, *R Russell Jones, [+]DO Haskard, JM Polak - Depts Histochemistry, *Dermatology and [+]Medicine, RPMS, Hammersmith Hospital, London, UK.

The cytokine-inducible cell adhesion molecules (CAMs), ICAM-1, ELAM-1 and VCAM-1 are important mediators of leukocyte attachment to vascular endothelium. In order to characterise and study the possible pathogenesis of cutaneous vasculitis we have investigated the immunocytochemical expression of these CAMs in skin biopsies from leukocytoclastic (LCV) and lymphocytic vasculitis (LV). The CAM immunoreactivity (IR) was correlated to the nature of the leukocyte infiltrate. Fourteen lesional and 7 non-lesional biopsies from patients with LCV and 10 lesional and 5 non-lesional biopsies from patients with LV were examined. Five control biopsies of normal skin were also examined. Keratinocyte ICAM-I IR was seen in 8 cases (80%) of LV and only 1 case (7%) of LCV. Endothelial ELAM-IR was found in 8 (57%) LCV and 5 (50%) LV biopsies. Endothelial VCAM-1 IR was seen in 2 cases (14%) of LCV and 5 cases (50%) of LV. VCAM-1 and ICAM-1 staining was significantly increased in perivascular mononuclear and dendritic cells in LV but not LCV. There was no significant difference in endothelial staining between the non-lesional and control biopsies. Neutrophils were significantly increased in LCV compared to LV whereas lymphocytes were more commonly seen in LV.

These results indicate that LCV and LV can be distinguished on the basis of preferential CAM expression. Furthermore, the pattern of CAM IR in LV is similar to that observed in the cell mediated immune (type IV hypersensitivity) response.

AUTOANTIBODIES IN ALOPECIA AREATA.

N.S. Calver, [1]S. Macdonald Hull, S.M. Parkin, [1]W.J. Cunliffe and V.A. Randall. Departments of Biomedical Sciences, University of Bradford, Bradford, BD7 1DP and [1]Dermatology, The General Infirmary, Leeds, LS1 3EX.

The aetiology of Alopecia Areata is unknown, although it is generally regarded as an auto immune disease. This study was designed to determine whether Alopecia Areata patients produce autoantibodies to components of the scalp hair follicle.

Frozen sections (5μm) were cut longitudinally from biopsies from the active edges of lesions in Alopecia Areata patients and from normal controls. Sections were incubated with either: saline, the subjects own serum, an anti-Human immunoglobulin fluorochrome conjugate, serum followed by anti-Human immunoglobulin fluorochrome conjugate, as a secondary antibody.

In 5 out of 6 control biopsies no appreciable increase in fluorescence was seen except in association with the inherent blood supply, identified by serial sections stained with Factor VIII antibody. In the other control biopsy fluorescence was seen around the follicular basement membrane of a catagen follicle, but not anagen follicles. In 15 of the 19 Alopecia Areata biopsies there was increased fluorescence associated with the outer root sheath. Subgroups of these individuals also showed an increase in fluorescence associated with the other keratinocyte related structures; the inner root sheath, hair shaft, epidermis, sebaceous glands and apocrine glands. In many of the Alopecia Areata biopsies fluorescence was also detected from incubation with the fluorochrome conjugate alone.

The differing reactions of the control and Alopecia Areata subject biopsies, to their respective serum, suggest the presence of autoantibodies to the hair follicle and other skin components in Alopecia Areata subjects. The fluorescence obtained with the fluorochrome conjugate alone indicates that autoantibodies were also deposited within the lesion before biopsy. These results indicate that autoantibodies may be involved in the aetiology of Alopecia Areata.

A DOSE-RESPONSE STUDY OF IRRITANT REACTIVITY IN SEBORRHOEIC DERMATITIS AND ATOPIC ECZEMA

N.C. Cowley and P.M. Farr, University Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne.

Although an increased susceptibility of the skin to the effects of physical and chemical injury is said to occur in patients with seborrhoeic dermatitis, this is not supported by experimental data. Irritant responses may be important however in the pathogenesis of seborrhoeic dermatitis, as enzymes from *pityrosporum ovale* can liberate fatty acids which may produce a local irritant effect. The susceptibility of the skin of patients with seborrhoeic dermatitis (n=13) to irritation was therefore investigated and compared to that of a group of normal patients (n=17) and patients with a history of atopic eczema (n=13), a disorder considered to be associated with an enhanced sensitivity to irritants.

Responses to 6 concentrations of sodium lauryl sulphate (SLS), (0.125%, 0.25%, 0.5%, 1%, 2% and 4%) applied to forearm skin under occlusion for 48 h, were assessed clinically and by laser Doppler flowmetry at 72 h after application. Dose-response curves were constructed in order to determine for each patient the dose of SLS required to produce an increase in blood flux of 0.125 volts ($D_{0.125}$), an objective measurement of response that corresponds approximately to just detectable erythema (clinical grade +1).

Visually assessed irritant reactions occurred at a significantly lower dose in patients with a history of atopic eczema (0.125%) compared to normal subjects (0.5%) ($p=0.006$), but there was no significant difference between normal subjects and patients with seborrhoeic dermatitis. There was a significant difference in $D_{0.125}$ between the three groups ($p=0.016$), with mean $D_{0.125}$ for patients with atopic eczema (0.33%) significantly lower than normal subjects (1.02%) ($p<0.01$, t test). The mean $D_{0.125}$ for patients with seborrhoeic dermatitis (0.49%) was also significantly lower than normal subjects ($p=0.05$).

Our study confirms the increased sensitivity to irritation in patients with atopic eczema previously demonstrated by other methods and shows for the first time that patients with seborrhoeic dermatitis also react abnormally to a topical irritant. The possibility that the increased reactivity to SLS demonstrated in patients with seborrhoeic dermatitis reflects a general inherent susceptibility to irritation and is involved in pathogenesis of the disease requires further study.

ULTRAVIOLET RADIATION INDUCES EXPRESSION OF P53 AND PROLIFERATING CELL NUCLEAR ANTIGEN IN HUMAN SKIN.

H.du P.Menagé, R.Dover[¶], C.Hobbs['], Y.Jayaram[¶], J.L.M.Hawk, P.M.McKee['], P.A.Hall['], Dpt of Photobiology, St John's Institute of Dermatology, St Thomas' Hospital, ICRF Histopatholoy Unit[¶], & Dept of Histopathology, UMDS, St Thomas' Campus['].

Detection of the p53 tumour suppressor gene product by immunohistochemical methods is generally held to be dependent on mutation in the gene which results in prolongation of protein half life and resultant accumulation of protein to detectable levels However alternative mechanisms may exist for stabilisation of the protein, p53 function perhaps being related to stress events such as DNA damage Proliferating cell nuclear antigen (PCNA) is a protein required for DNA excision repair and expression has been noted following ultraviolet (UV) irradiation in vitro We therefore studied expression of p53 and PCNA following UV irradiation

The forearms of two volunteers was exposed to 1 5 minimal erythema doses of solar simulated radiation (Kratos filtered Xenon arc solar simulator) Biopsies were taken at 1, 2, 4, 8, 24, and 48 hours and 2 weeks following irradiation One control biopsy was taken from the contralateral forearm Expression of p53 was assessed using an immunoperoxidase technique with antibodies CM-1, Do-1, 1801, and 240, and to PCNA with antibody PC10 (D P Lane)

p53 immunoreactivity was detected at 4 hours with a peak at 24 hours, when 10% of basal keratinocytes stained positively, and had diminisheed to non detectable levels at 2 weeks No staining was detected with the 240 antibody, which detects conformational change in the protein. PCNA immunoreactivity showed similar temporal and spatial expression (although controls showed occasional nuclear staining on basal cells)

Transient induction of p53 was also seen in vitro using cultured normal human keratinocytes (irradiated with a San Gabriel UVGL58 multiband mineralight lamp)

Ultraviolet exposure in physiological doses can induce transient p53 expression at times when PCNA expression is also present This is compatible with a role for p53 in the cellular response to DNA damage

PERCUTANEOUS PENETRATION FROM A LOW DOSE SUPERSATURATED HYDROCORTISONE ACETATE FORMULATION

R Marks, PJ Dykes, J Gordon,* G Hanlan* & AF Davis*, Department of Dermatology, UWCM, Cardiff CF4 4XN and *SmithKline Beecham, Consumer Brands, Weybridge, Surrey

Percutaneous penetration may be enhanced by supersaturation of the drug concerned The use of low concentration supersaturated formulations offers the opportunity of minimizing local overdosing whilst maintaining efficacy In this study the use of supersaturated low doses of hydrocortisone acetate (HA) has been studied both in vitro and in vivo In an in vitro model, using a 0 04mm polydimethylsiloxane membrane under finite dose conditions, a 0 02%w/w 8x supersaturated HA gel has been compared to a standard 1% HA formulation Significantly improved release was observed·

Time·	1 hour	2 hour	4 hour	6 hour	7 hour
0 02% HA gel	0 006mg	0 009mg	0 022mg	0 032mg	0 035mg
1% HA cream	0 004mg	0 004mg	0 006mg	0 013mg	0 012mg

Supersaturated (HA) has also been investigated in human volunteer subjects using the vasoconstrictor (VC) test and an irritant dermatitis model. Four treatments have been compared; 1% HA cream, E45 cream, 0 02% 8x supersaturated HA gel and placebo gel In the VC test, 18 subjects were treated with 5mg over an area of 7x7mm on the forearm Sites were occluded for 8 hours and pallor assessed at 0 5,1,1.5,2 and 16 hours to obtain a cumulative score Irritant dermatitis was induced by solutions of sodium lauryl sulphate (2x minimal irritant dose) for 24 hours under occlusion to the forearms of 16 subjects At 48 and 72 hours the sites were assessed for erythema using 10cm visual analogue scales (VAS) and an erythema meter.

	1% HA	E45	0 02% HA	Gel	No Tr
VC Score	15 8	5 3	21 9	4.20	4 9
VAS 48h	2 14	9.93	2 10	5 43	9 88
VAS 72h	8 05	9 83	8.16	8.59	10 0
E Meter 48h	-0 25	6.81	0 44	2 50	5 38
E Meter 72h	4 75	7.38	5 25	5 25	7 50

The VC test showed that both active treatments were statistically different from their placebos and untreated site There was no difference between active treatments. Similar results were found with VAS scores at 48h but not at 72h The erythema meter readings at 48h showed significant differences with 1% HA cream but the result for 0 02% HA gel just failed to achieve significance. The results of these studies support the hypothesis that low dose supersaturated formulations of comparable efficacy to standard corticosteroid formulations can be prepared

A COMPARISON OF THE EFFECTS OF KETOCONAZOLE AND TERBINAFINE ON THE ω-OXIDATION OF 12-HETE TO 12,20-DIHETE BY CYTOCHROME P-450 IN NEUTROPHILS.

S.C.Fox and B.R.Allen. Department of Dermatology, University Hospital, Queens Medical Centre, Nottingham NG7 2UH.

The antifungal agents ketoconazole and terbinafine exert their effect by inhibition of ergosterol synthesis. Ketoconazole achieves this by inhibiting lanosterol 14-demethylase which is a cytochrome P-450 mediated enzyme whereas terbinafine is more effective at inhibiting a non-cytochrome enzyme, squalene epoxidase.

In human neutrophils 12-HETE, an important component of the 12-Lipoxygenase pathway, is converted by ω-oxidation to 12,20-DIHETE by a cytochrome P-450 enzyme in the cell membrane. This ω-oxidation process is an important step in the breakdown of 12-HETE, a potent stimulant of chemotaxis. The study was designed to make a direct comparison of the effects ketoconazole and terbinafine on this enzyme.

Neutrophils (5×10^6 cells/ml) from the same donor were incubated at 37°C for 2 minutes with a range of concentrations of either ketoconazole or terbinafine or their controls prior to the adddition of 12(S)-HETE (6 25µM) After incubation for a further 10 minutes the reaction was terminated by mixing the aliquot with an equal volume of HPLC buffer (78% Methanol), plunging into ice and microcentrifuging at 1300rpm to remove the cells. The 12,20-DIHETE produced was measured in duplicate by RP-HPLC. Inhibition was calculated as a percentage of the control value for each compound Mean results from five seperate donors were calculated.

Potent inhibition of 12,20-DIHETE production by ketoconazole was seen with an IC-50 value of 4.6µM. Slight inhibition of 12,20-DIHETE production was obtained with terbinafine though the IC-50 value was >100µM, the maximum concentration studied.

Cell viability was determined by incubating cells with the highest concentrations of each drug studied for 12 minutes at 37°C followed by 10 minutes at room temperature with Trypan Blue Solution. Cell viability of >90% was shown with concentrations below 5×10^{-4}M of ketoconazole and below 10^{-4}M of terbinafine.

Our results show that terbinafine has little effect on the cytochrome P-450 enzyme in human neutrophils whereas ketoconazole is a potent inhibitor of this enzyme. Our observations may be of clinical importance when considering the toxicity of these drugs in human use.

MALASSEZIA FURFUR SPECIFIC IgG SUBCLASSES IN PATIENTS WITH SEBORRHOEIC DERMATITIS AND CONTROLS

A Fruin, H R Ashbee, E Ingham, [1]K T Holland and [2]W J Cunliffe, Depts of Immunology, [1]Microbiology and [2]Dermatology, (Leeds Foundation for Dermatological Research), University of Leeds, Leeds

Malassezia furfur is considered to be the aetiological agent in seborrhoeic dermatitis Previous investigations have shown higher antibody titres to M furfur in patients compared to controls It has been suggested that this represents a typical response to antigenic stimulation However normal antibody titres may mask an IgG subclass imbalance and imbalanced IgG subclass patterns with increased IgG4 have been recorded in allergic diseases and chronic mucocutaneous candidiasis In order to determine whether an imbalance of M furfur specific IgG subclasses was present in seborrhoeic dermatitis patients the levels of IgG1 - IgG4 specific for 3 serovars of M furfur (A, B & C) were determined in 10 patients and 10 age and sex matched controls

A transferrable solid phase ELISA for detection of M furfur specific IgG subclasses was designed and fully validated IgG subclass titres specific to M furfur A, B and C were recorded as the highest serum dilution giving an optical density 0 05 units above a negative control serum The mean reciprocal IgG subclass titres are given below

M. furfur serovars	IgG subclass	Mean of reciprocal titres Patients	Controls
A	1	20 0	9 6
	2	30 4	21.2
	3	32.8	10 0
	4	23 2	20 8
B	1	50 0	20 8
	2	45 2	29 6
	3	30 4	18 0
	4	56 8	41 6
C	1	47 2	24 4
	2	30 4	27.2
	3	30 8	14 0
	4	28 0	22 4

Results analysed by 3-way analysis of variance, revealed no significant variation amongst the IgG subclasses, a significant variation amongst patients and controls ($p<0\ 001$) and a significant variation amongst serovars ($p<0\ 001$). Perusal of data indicated that these results were due to higher IgG subclass titres overall in the patient group and higher IgG subclass titres to M furfur serovars B and C compared to A Individual means were compared by calculating the minimum significant difference (38 8, $p<0\ 05$) which revealed no significant differences These data indicated that patients with seborrhoeic dermatitis were more sensitised to M furfur than controls There was no evidence of an IgG subclass imbalance in patients It was of interest that IgG3 and IgG4 titres were similar to IgG1 and IgG2 titres. Since the ratio of IgG subclasses in normals is 16 6 1 5 1, for IgG1-4 respectively, specific IgG subclasses to M furfur were biased towards IgG3 and IgG4, indicating chronic exposure to the yeast in patients and controls

ISOLATION AND MAINTENANCE OF THE HUMAN PILOSEBACEOUS DUCT: 13-CIS RETINOIC ACID ACTS DIRECTLY ON THE DUCT *IN VITRO*

R. Guy, C C.Ridden, [1]J H Barth and G T E. Kealey Department of Clinical Biochemistry, University of Cambridge and [1]Department of Chemical Pathology and Immunology, University of Leeds

Acne vulgaris is a disease of the human pilosebaceous unit Although the strongest predictor of the severity of acne is the rate of sebum secretion, acne is not a disease of the sebaceous glands themselves Rather, it appears to be a disease of the pilosebaceous duct and is characterised by hyperproliferation, hyperkeratinisation and and abnormal desquamation of the duct cells It remains unclear if the abnormalities of the pilosebaceous duct in acne are primary or secondary to seborrhoea and it is also unclear if 13-cis retinoic acid achieves its therapeutic effect by acting directly on the duct, or indirectly by reducing the sebum secretion rate. To help answer these questions we have isolated the human pilosebaceous duct, shown it is viable on isolation and maintained it for a week in the presence or absence of 13-cis retinoic acid

Redundant human female facial skin was obtained from cosmetic surgical procedures. Layers of skin were removed by means of a keratome The ducts were identified in the dermal layer using a dissecting microscope and isolated by microdissection Isolated pilosebaceous ducts were maintained in supplemented Williams E medium Where appropriate the medium was supplemented with 1 µM 13-cis retinoic acid.

Histlogical sections of the freshly isolated duct showed that it had maintained its *in situ* architecture exhibiting a stratified, squamous, keratinising epithelium. On maintenance for seven days the duct loost its stratified squamous pattern and appeared to rupture causing an apposition and flattening of the duct cells onto an empty lumen Ducts maintained in the presence of 13-cis retinoic acid retained their architecture far better, which may be attributed to a reduction in cell division

The rate of DNA synthesis, as shown by [methyl-^{3}H] thymidine uptake, showed a fresh value of 9 09±1 47 fmols/µg protein/hour (n=10) On maintenance for seven days the rate fell to 6 79±0 98 fmols/µg protein/hour (n=5) The addition of 1 µM 13-cis retinoic acid to the maintained ducts caused a further significant fall to 3 43±0 51 fmols/µgprotein/hour The rate of uptake of [methyl-^{3}H] thymidine and [U-^{14}C] leucine was linear over eight and a half hours

The pattern of keratin synthesis in freshly isolated ducts, as shown by one dimensional 10% polyacrylamide gels of keratins incubated with ^{35}S-methionine, showed the keratins nos 1,5,10 and 14 to be present, all of which are found in the human epidermis

The [methyl-^{3}H] thymidine pattern of autoradiography showed the region of cell division of the fresh duct to be the basal cells of the periphery This is in agreement with previous *in situ* results It is difficult to discern the region of cell division of ducts maintained for seven days in the presence or absence of 13-cis retinoic acid due to the experimental procedures but the degree of graining for each is consistant with the rate of DNA synthesis already described

In conclusion, we have isolated and maintained the human pilosebaceous duct, and demonstrated that 13-cis retinoic acid acts directly at the level of the duct.

KERATIN EXPRESSION IN DYSTROPHIC HAIR AND NAILS IN ALOPECIA AREATA.

D. Holland, S. Macdonald Hull, G. Roberts, W.J. Cunliffe. Dept. of Dermatology, Leeds General Infirmary

The principal keratins found in hair and nail are two type I (Mr 45 and 42kD) and two type II (Mr 62 and 61 kD) polypeptides. Whether these keratins are synthesised in this form or are modified from larger precursors during growth and differentiation has not yet been determined. The features of alopecia areata (AA) are dystrophic hair, depigmented hair or complete loss of hair and in many cases pitting and ridging of the nails. The aim of this study was to determine whether these abnormalities were reflected in a modified keratin profile. The hairs were cut from the edge of a patch of AA or were clinically dystrophic; all the nail clippings were taken from dystrophic nails. 16 hair samples and 40 nail samples from AA patients thus obtained and similar samples from 5 control subjects were ground in liquid nitrogen and extracted in 10% (w/v) SDS, 5% (v/v) mercapto-ethanol, 0.05M Tris-HCl pH 6.8 buffer at 80° for 30 min, samples were homogenised and sonicated. After centrifugation, the supernatants were analysed by gradient SDS-PAGE and scanning densitometry. Four keratins of Mr 61kD, 60kD, 45kD and 42kD were present in all hair and nail samples from both normal subjects and AA patients. Additional keratins, possibily of epidermal origin, of Mr 59 kD, 51 kD and 49 kD were also present in the nail samples. These results suggest that hair and nail keratin expression is normal in AA and that there appears to be no imbalance between these structural proteins. Gene analysis of hair keratins is now required to reveal whether mutations in these proteins are responsible for hair and nail dystrophy.

IN VITRO CULTURE OF SHEEP WOOL AND RAT VIBRISSA FOLLICLES.

[1]P I.Hynd, [2]K.A.Gunn, [2]A J Reynolds and [2]C.Jahoda.
[1]Dept. of Animal Sciences, University of Adelaide, South Australia. [2]Dept. of Biological Sciences, University of Durham, Durham.

The method described for human follicle culture has been tested on two contrasting follicle types. Wool follicles from the neck region of three sheep (Suffolk crosses) were dissected singly, or as small groups. Rat vibrissa follicles were isolated and grown as complete structures, or with the upper half of the follicle removed. Follicles were grown in Williams medium as previously described(1). Of more than thirty wool follicle specimens, over half showed fibre lengthening after seven days incubation with the longest fibre 2mm (equivalent to 0.26mm/day) - but around 1mm was more common. Follicles cultured in small groups were as likely to show fibre extension as single ones. Dark field microscopy, and routine histology confirmed the presence of a fibre producing bulb at the base of most follicles. This suggested that genuine growth had occurred and that it could be prolonged if required. Of eighty eight cultured vibrissa follicles, seventy one showed fibre length increases after seven days - all 34 cut follicles showed the effect. However periodic measurements revealed length increases to be extremely variable and erratic: some follicles had a delayed response, and rates of fibre increase varied from approximate *in situ* values of 0.75mm/day to below 0.1mm day. Most length increases did not approach normal values and almost all extension had stopped at seven days Histology revealed a variety of bulb abnormalities including evidence of repeated attempts to grow hair, and outer root sheath extension/hyperproliferation. This culture methodology is clearly applicable to wool follicles, whereas its use for vibrissa follicle work may be specialized.

1Philpott M.P, Green M.R., Kealey T. Human hair growth *in vitro*. J Cell Sci 1990;97, 409-418.

VARIATION IN THE EXPRESSION OF ADHESION MOLECULES WITHIN THE PSORIATIC PLAQUE.

A.M. Layton, C. Morris, [1]E. Ingham, M.G. Goodfield, W.J. Cunliffe. Dept. of Dermatology, Leeds Foundation for Dermatological Research. [1] Dept. of Immunology, Leeds University.

The expression of ICAM-1, ELAM-1 and VCAM-12 is known to be upregulated in psoriasis. The aim of this study was to assess any variation in the expression of these adhesion molecules within different parts of the psoriatic plaque.

Fifteen patients with untreated psoriasis were included in the study. Laser flowmetry was applied to the plaques in order to define the developing and resolving edges (JID 92: 782-5). Four millimetre punch biopsies were taken from these edges, as well as from the centre of the lesion. The expression of ICAM-1, ELAM-1 and VCAM-1 was assessed using standard immunohistochemical techniques.

Results demonstrated increased expression of all three adhesion molecules within the psoriatic plaque. Blood vessels were positive for VCAM-1 and

ELAM-1 as was the dermal infiltrate. No differences were demonstrated with respect to VCAM-1 and ELAM-1 expression, except in the dermis where there was upregulation of VCAM-1 in the central plaque compared to both edges. There was positive expression of ICAM-1 within the epidermis, dermis and around vessels. This was increased at the resolving epidermal edge when compared to the developing and central areas.

Previous data suggests that ICAM-1 expression promotes lymphocyte adhesion. However, this work supports the hypothesis that ICAM-1 expression on psoriatic keratinocytes may also occur as a result of cellular infiltration and activation. Such upregulation may occur as a result of cytokine release from activated T-cells.

MORPHOLOGICAL CHANGES OF NEURAL AND VASCULAR PEPTIDES IN HUMAN SKIN SUCTION BLISTER LESION

G Terenghi, XH Gu, *PE Purkis, JM Polak, *I Leigh
Histochemistry Dept, Hammersmith Hospital and *Skin Tumour Laboratory, Royal London Hospital, London.

Suction blister injury is an experimental model for the investigation of dermal/epidermal interaction and possible derangement in disease. An extensive fibre network can be stained using antisera to the pan-neuronal marker protein gene product 9.5 (PGP) and the sensory neuropeptide calcitonin gene-related peptide (CGRP), while microvessels are identifiable with the endothelial marker von Willebrand's factor (vWf) and the peptide endothelin (ET). These immunoreactivities (IR) have been studied by quantitative immunohistochemistry in human skin at different times after suction blister, in order to investigate the reactive and repair processes in injury. PGP and CGRP-IR nerves showed a numerical increase up to 6 hours (h) after lesion, which was followed by a decrease that lasted until 48h. An increase of both nerve types was observed at the blister edge from 72h which spread to the centre of the blister area and lasted until 8 days (d). Similarly vWf and ET-IR capillaries increased numerically between 0h and 6h, to decrease then at 12h and 18h. From 21h onwards both endothelial IR showed a steady increase which lasted until 8d. Generally the numerical decrease was accompanied by weak staining intensity, which reverted to strong staining at later time points. These results would suggest that there is a reactive process for all IR elements in early stages of injury, followed by changes indicative of neuronal and endothelial damage (depletion phase) and subsequent repair process.

REINNERVATION PATTERNS IN SKIN FOLLOWING DENERVATION AND ISCHAEMIA: THE ROLE FOR ANGIOGENESIS.

S Manek[1], C Shurey[1], G. Terenghi[2], J.M. Polak[2] and C.J. Green[1], [1]Section of Surgical Research, CRC, Harrow and [2]Dept. of Histochemistry, RPMS, London.

A study of the patterns of reinnervation and revascularization in autografted adipomyocutaneous flaps following denervation and ischaemia was undertaken with an aim to discern influencing factors and to provide information for clinical skin transplantation. Flaps were either partially (Group 1) or totally denervated (Group 2). In Group 2, the flaps were either rendered transiently ischaemic for 30 min (Group 2A) or grafted without vascular anastomosis resulting in prolonged ischaemia (Group 2b). The flaps and surrounding skin were examined at 4, 7, 12 or 24 days using antisera to protein gene product (PGP 9.5 - a panneural marker), calcitonin gene related peptide (CGRP - for sensory nerves), C-flanking peptide of neuropeptide Y (CPON - for adrenergic nerves) and to von Willebrand factor(VWF - an endothelial marker).

Within surrounding skin, early increases in PGP-immunoreactive (PGP-IR), CGRP-IR and CPON-IR nerve fibres were seen. These were mild in skin immediately surrounding Group 1 flaps, moderate in skin distant to Group 2A flaps and moderate to marked in all areas surrounding Group 2B flaps. IR nerve fibres in the flap bases appeared later. At 24 days, PGP-IR and CGRP-IR nerve fibres were seen in all flaps, following earlier complete depletion of immunoreactivity. With VWF, an increase in vascularity (granulation tissue) was indicated in surrounding skin, flap beds and the pedicle, particularly in partially denervated flaps.

Reinnervation took place from surrounding skin prior to that from the base, with sensory fibres appearing first, while angiogenesis preceded reinnervation. Denervation was found to be more detrimental to cutaneous innervation than any degree of ischaemia.

THE DERMO-EPIDERMAL INTERFACE IN LICHEN PLANUS AND LUPUS ERYTHEMATOSUS.

P Marren, D deBerker, F Wojnarowska, [1]MG Davies, [2]IM Leigh, SM Burge. Department of Dermatology,The Slade Hospital Oxford, [1]Greenbank Hospital, Plymouth and the [2]London Hospital.

Biopsies from involved and uninvolved skin of eight patients with lichen planus (LP) and involved skin of eight patients with lupus erythematosus (LE) and three normal controls were probed using a variety of monoclonal antibodies. Epidermal associated antigens were probed using Mabs to G71 (a^{6} b$_{4}$ integrin), GB3 (BM 600), and polyclonal sera to the 220 kDa bullous pemphigoid (BP) antigen. Dermal associated antigens were probed using Mabs to collagen type IV, L3d, LH7.2, (collagen type VII), GDA J/F3 (collagen type VII associated), LH 24 and LH39 (target antigens unknown) and polyclonal antibodies to the antigen of linear IgA disease (LAD), laminin and fibronectin.

Results with LP sections showed the uninvolved skin to be indistinguishable from normal controls. In involved skin, expression of the epidermal associated antigens, a^{6} b$_{4}$ integrin and BP was impaired in all sections. We frequently identified damaged keratinocytes overlying gaps in the BMZ. In contrast, the dermal associated antigens, collagens type IV and VII and laminin became thickened in response to the inflammatory process. Reduplication of the BMZ was seen in four of eight sections and chunky fronds extended down into the papillary dermis in all. In LE sections a similar loss and disruption of the epidermal antigens was seen. The structural collagens were thickened with reduplication in three of eight sections. Chunky fronds extending into the dermis were more pronounced in this disease. There is enhanced expression of fibronectin in LP and LE lesions, most dramatic in the LE sections which may correlate with the scarring tendency in LE.

INHIBITION OF NICKEL-INDUCED CONTACT ALLERGY BY TOPICAL CHELATING AGENTS AND ANTI-OXIDANTS.

A.A. Memon, M.M. Molokhia, P.S. Friedmann, Department of Dermatology, University of Liverpool.

Allergic contact sensitivity to nickel is a very common problem and can cause significant morbidity with chronic eczematous dermatitis. Although avoidance of nickel is the ideal therapy, this is often impossible. The routine treatment is topical steroids, but their use is limited due to the risk of side-effects. We have therefore prepared "barrier" creams containing either chelating agents - Iodochlorohydroxyquinoline (vioform) or EDTA or anti-oxidants - vitamin C, vitamin E; 1% Hydrocortisone (HC) was included as an anti-inflammatory control. Nickel sensitive subjects were challenged for 48 hours with 20-

pence coins coated with each cream. In a pilot study nickel containing coins elicited uniform and reproducible contact allergic reactions and thus "model" normal exposure to metal containing objects. To see whether a layer of Yellow, soft paraffin (YSP) vehicle applied between coin and skin can impair the responses, coins were coated with 50, 100, 150 or 200 mg of YSP compared with no YSP. There was no reduction in the magnitude of reactions with in the range of quantity tested. Vioform down to 3% completely abolished the allergic reaction in all 26 nickel sensitive subjects tested. Below 3% the effect was reduced. EDTA (15%) had a lesser effect reducing the reaction in 40% of the subjects. Vitamin C (10%), vitamin E (10%) and 1% HC were without any effect. In a clinical use study, we compared the effects of a commercially available cream containing 3% vioform and 1% HC (vioform HC), with 1% HC cream in subjects with bilateral nickel dermatitis. Sites treated with vioform-HC showed clinical improvement in all nine subjects tested. Therefore vioform can be used as a "barrier" cream to block the allergenic effects of nickel in sensitive individuals.

HYPERPROLIFERATIVE KERATIN EXPRESSION IN CUTANEOUS MESOENDEMIC SAVANNAH ONCHOCERCIASIS IN NORTHERN NIGERIA

R C Patel[6], M E Murdoch[1 4 5], L. Churchill[7], B R Jones[3], A. Abiose[1 2], I M. Leigh[7].

WHO Project on immunopathology of onchocerciasis ID 890134 based at the Guinness Ophthalmic Unit, Ahmadu Bello University[1], the National Eye Centre[2], Kaduna, Nigeria and Institute of Ophthalmology, London[3]; and the Institute of Dermatology[4], Imperial College of Science, Technology & Medicine, London[5], Clinical School, Addenbrookes Hospital, University of Cambridge[6] and Department of Experimental Dermatology, London Hospital Medical College[7].

Onchocerciasis, a filarial infection caused by *Onchocerca volvulus*, is endemic across tropical Africa. Individuals living in mesoendemic onchocercal villages, Kaduna State, Northern Nigeria underwent skin examinations within the context of a community based placebo-controlled trial of the new microfilaricidal drug ivermectin

Patients were selected for skin biopsy in the following groups. Acute Papular Onchodermatitis (APOD, n=7), Chronic Papular Onchodermatitis, (CPOD, n=10), Lichenified Onchodermatitis (LOD, n=5), Atrophy (ATR, n=4), Depigmentation (DPM, n=5) and Normal Skin High Microfilarial Load (NSHMF, n=5) using our recently developed clinical classification system (Murdoh, ME, Hay RJ, Ramnarain N, Lucas S, Jones B, Abiose A. A clinical classification and grading system of the changes in onchocerciasis and histopathological findings. *Br J Dermatol* 1990;123(37 Suppl):28) Biopsies from Nigerians living in a rural area non-endemic for onchocerciasis served as controls (NONEND, n=5). Individuals received a single dose of ivermectin or placebo and a repeat biopsy was taken one to three weeks later in the following patients.- 4 APOD and 7 CPOD. 3mm punch biopsies were snap frozen in liquid nitrogen and transported to the UK. The keratin expression in these specimens was examined blind using a standard immunofluorescence technique

In 5/5 LOD, 9/10 CPOD and 2/7 APOD the hyperproliferative keratin 16 (K16) was expressed suprabasally, but this was absent in 4/4 ATR, 5/5 DPM, 5/5 NSHMF and 5/5 NONEND. The positive staining in the APOD and CPOD specimens was localised to the papule region of the biopsy. Of the patients who expressed K16 pre-dosing, expression persisted in 4/5 CPOD post placebo and 1 CPOD post ivermectin. This latter patient continued to express K16 even at 10 weeks post treatment. The single APOD patient who lost K16 expression post ivermectin had cleared clinically by the time of the follow-up biopsy

Keratin expression in the epidermis is closely related to the proliferative state - hyperproliferative keratins are seen in the suprabasal epidermis in wound healing and psoriasis. The results suggest that the hyperproliferative phenotype is induced in certain clinical groups by filarial infection of the dermis and persistance or loss of K16 expression parallels the clinical course.

Acknowledgments This investigation received financial support from the UNDP/World Bank/WHO/ Special Programme for Research and Training in Tropical Diseases (ID 870456 and 890134), the Leverhulme Trust and the Royal Commonwealth Society for the Blind. MEM is supported by the Wellcome Trust.

MAJOR HISTOCOMPATIBILITY ANTIGENS AND SKIN CANCER IN RENAL ALLOGRAFT RECIPIENTS.

JM McGregor[1a],V Venning[1b],R Morris[3a],P Morris [2b], K Welsh[2a], DM MacDonald[1a]. Depts. of Dermatology[1], Tissue Typing[2] and Public Health Medicine[3], Guy's Hospital, London SE1 9RT[a] and John Radcliffe Hospital,Oxford[b].

743 caucasian patients from two renal transplant centres were assessed to determine a possible association between HLA-antigens and non-melanoma skin cancer following renal allograft transplantation. Of 72 patients found to have skin cancer, 38 had squamous cell carcinomas (SCC), 19 had basal cell carcinomas (BCC) and 15 had both.

Using Fishers exact test to compare HLA frequencies in the transplant population with and without skin cancer, HLA-DR1 was found at a lower frequency in patients with SCC and those with BCC compared to patients without skin cancer ($p=0.07$ and $p=0.06$ respectively). No association was found for any other class I or class II major histocompatibility (MHC) antigen and skin cancer. Life time risk analyses performed on these data indicate a possible protective effect against skin cancer associated with HLA-DR1, rather than with HLA-A11,as previously suggested in renal allograft recipients. This observation is of particular interest since paradoxically, an increased incidence of HLA-DR1 has been consistently observed in caucasian patients with skin cancer in the general (non-immunosuppressed) population.

The mechanisms which underlie an association between certain HLA antigens and an increased incidence of cancer are not currently understood. Nonetheless, the observation that HLA-DR1 may be positively associated with skin cancer in the general population but protective in renal allograft recipients suggests that this MHC locus may play a fundamental role in determining the host effector response to squamous cell tumour antigens. An alternative, and not exclusive possibility, is that squamous cell tumour antigens are distinct in immunosuppressed and non-immunosuppressed patients, perhaps reflecting a different aetiology.

LOCALISATION OF EPSTEIN BARR VIRUS DNA IN ATYPICAL LYMPHOCYTES OF CUTANEOUS B CELL LYMPHOMA BY IN SITU HYBRIDISATION.

JM McGregor, QL Lu[1], FE Cotter[2], DA Levison[3], DM MacDonald. Depts. Dermatology and Histopathology[3], Guy's Hospital,SE1 9RT and ICRF, St Bartholomews Close, EC1[2] and Lincolns Inn Fields[1], W1, London UK.

Established data now confirms a close association between Epstein Barr Virus (EBV) infection and peripheral B cell lymphoma in renal allograft recipients. To date cutaneous lymphoma has not been described in this population however. This study examines four cases of cutaneous lymphoma diagnosed in a transplant population of 673 patients. Primary, recurrent and metastatic tissue (where available) was examined by light microscopy, immunocytochemistry, polymerase chain reaction (PCR) and southern blotting and by in situ hybridisation.

In three primary lymphomas and in one case of recurrent lymphoma, each expressing the Ki-1 antigen, a clonal population of B lymphocytes was identified by immunoglobulin gene analysis. In each of these tumours EBV DNA was identified by PCR and Southern blot analysis. Localisation of EBV DNA to large atypical lymphocytes was further demonstrated by in situ hybridisation in three of these four tumour specimens. In one further primary lymphoma, and in metastatic tumour from this patient, T cell receptor gene re-arrangement studies demonstrated a clonal population of T lymphocytes. EBV DNA was not identified in these tumour specimens either by PCR or in situ hybridisation.

The demonstration of EBV DNA in malignant lymphocytes, but not in surrounding reactive lymphocytes, in three cases of cutaneous B cell lymphoma suggests an oncogenic role for herpes virus' in the development of these tumours in transplant recipients. A possible similar role for EBV in the aetiology of cutaneous B cell lymphoma in the general (non-immunosuppressed) population requires further investigation.

HEMIDESMOSOME ONTOGENY IN HUMAN FETAL DIGIT SKIN

J.R McMillan and R.A.J. Eady, Dept. of Cell Pathology, St John's Institute of Dermatology, St Thomas's Hospital, London.

Hemidesmosomes (HDs) consist of discrete electron-dense thickenings or plaques on the inner plasma membrane that connect epidermal keratinocytes to the underlying dermis. Various antigenic components of HDs have been identified as early as 9 weeks' estimated gestational age (EGA) in some sites in the body. Their appearance coincides with the formation of an intermediate layer of cells between the periderm and the basal layer. The question arises whether HDs suddenly appear as complete assembled structures or accumulate and develop over a period of time. This information might help with the understanding of junctional epidermolysis bullosa which is characterised by an abnormality of HDs, also present during fetal development. Samples of digital skin from eight normal fetuses of 9 or 15 weeks EGA were processed for routine electron microscopy. High magnification electron micrographs were taken to analyse 40 μm of continuous basement membrane. HDs were then counted and the density calculated using a modification of an established method (Tidman MJ, Eady RAJ. J Invest Dermatol 1984; 83· 448-453.) Each HD was also scored for three morphological characteristics: presence of a sub-basal dense plate (SBDP), intermediate filament (IF) association and presence of anchoring filaments (AFs). There was a significant increase ($p=0.0012$) in HD counts between 9 and 15 weeks with counts of 20.6 / 40μm (± 3.81 SEM) and 95.8 / μm (± 8 4 SEM) respectively. Previous data from adult knee showed slightly lower HD values than the 15 week fetal skin, possibly due to regional influences. In addition there was a slight increase with time in the percentage of HDs exhibiting IF association [5.5 (± 3.7 SEM) vs. 24.49 (± 7.0 SEM)($p=0.15$)] which also coincided with the appearance of a proximal density above the plaque. Between 9 and 15 weeks no variation was found in the percentage of HDs with AFs [3.6 (±2.3 SEM) vs. 4.7 (±3.3 SEM)] or SBDPs [24.5 (± 5.6 SEM) vs. 23.8 (±4.3 SEM) $p=0.9$]. In summary, HDs are first formed in small numbers without prominent IF filament contact and the IF association occurs sometime between 9 and 15 weeks via the proximal density. The early appearance of SBDPs and AFs suggest that these components are critical in the formation of HDs, supporting previous data derived from cell culture and wound healing experiments.

ABNORMALITIES IN HAIR FOLLICLE MELANOCYTES FROM CLINICALLY NORMAL AREAS OF ALOPECIA AREATA SUPPORT A ROLE FOR MELANOCYTES IN THE AETIOLOGY OF THIS DISEASE.

M Nutbrown, [1]S Macdonald-Hull, N.S Calver, [1]W J Cunliffe & V A. Randall, Departments of Biomedical Sciences, University of Bradford, BD7 1DP & [1]Dermatology, The General Infirmary, Leeds, LS1 3EX

Although alopecia areata is thought to be an auto-immune disorder the aetiology is unknown Melanocytes may be involved since white hair may be spared and regrown hair may be unpigmented, abnormal hair bulb melanocytes and other pigmentary abberations have also been detected in active lesions of alopecia areata (Tobin et al J Invest. Dermatol 94:803-807, 1990)

Since examination of the sub-clinical condition detected in **clinically normal** hair bearing regions of alopecia areata scalps (Macdonald Hull et al J Invest Dermatol 96·637-681, 1991) may reveal important aetiological information, the ultrastructure of melanocytes and melanosomes has been investigated in follicles from these areas Scalp biopsies were taken from both active edge and **clinically normal** hair bearing regions of scalps of six alopecia areata patients aged 15 to 58 years, together with five normal controls, and routinely processed for transmission electron microscopy

Melanocytes from control follicles had many dendrites and contained numerous melanosomes There was some cytoplasmic vacuolation and hyperchromaticity of nuclei but distribution of pigmentary granules was well organised, control follicles showed only slight signs of pigmentary components in the dermal papilla Abnormal melanocytes and unusual distribution of melanosomes were seen in hair bulbs from active areas of alopecia areata, as previously reported In **clinically normal** hair follicles melanocytes also showed extensive vacuolation and nuclei were often hyperchromatic, highly indented and surrounded by a lucent border Melanophages were present in the dermal papillae and basal stalks of **clinically normal** follicles and giant complexes of clumped pigmentary granules were seen in dermal papilla cells

The degenerated appearance of melanocytes in the hair matrix and the presence of melanophages and giant spherical complexes of pigmentary granules in the dermal papillae of **clinically normal** hair follicles from alopecia areata patients concurs with the hypothesis of melanocytic involvement in alopecia areata and supports the concept of a sub-clinical condition of the disease

A QUANTITATIVE STUDY OF THE DIFFERENTIAL EXPRESSION OF ALPHA-SMOOTH MUSCLE ACTIN IN DERMAL CELL POPULATIONS OF FOLLICULAR AND NON-FOLLICULAR ORIGIN.

[1]A.J Reynolds, [2]C.Chaponnier, [1]C.Jahoda and [2]G Gabbiani. [1]Dept. of Biological Sciences, University of Durham, Durham.
[2]Dept of Pathology, University Medical Centre, Geneva

Alpha-smooth muscle actin (ASMA) is an actin isoform found in smooth muscle cells, myofibroblasts and a specific region of follicular dermal sheath tissue *in vivo*. The ASMA content of 4 adult hair follicle-derived dermal cell populations (papilla and 3 levels of sheath), was compared to that of adult and 4 day rat fibroblasts initiated from mystacial, ear and footpad skin. Assessment involved: (a) double immunofluorescent staining (1000 cells counted in 10 random fields), (b) comparative Western blot estimations and, (c) determinations from 2D-electrophoretic gel analysis (involving DNA, protein and actin isoform calculations) - (a) incorporated data from at least 6, and (b) and (c) from at least 4, independently prepared batches of cells. Quantification in (b) and (c) included scanning with a computerised laser beam densitometer linked by a digital converter analogue to an IBM AT compatible microcomputer. The resultant data from all three methods was highly correlative. All sheath cultures were morphologically similar, but while those from the upper regions contained fewer ASMA positive cells (50%) than end bulb-derived populations (80%), cultures of lower sheath (from between the two) consistently had the highest proportion (98%). The variable but low levels of ASMA expression observed in the adult skin fibroblasts (below 10%), could be directly related to the hair follicle density in the site of their origin (mystacial > ear > footpad) The differential expression of ASMA by follicular populations *in vivo* and *in vitro*, provides an insight into the specialist nature of these cells and their derivation. Moreover, the proposition that follicular dermal cells may represent unappreciated constituents of all fibroblast populations derived from hair-bearing skin, has wider implications for cell culture methodology and wound-healing research.

INDUCTION OF KERATINOCYTE PROLIFERATION IN VIVO BY INTERFERON-GAMMA (IFN-γ). EL Ross, JNWN Barker, [1]JR Goodlad, EL Ross, [2]C-W Yu, RW Groves and DM MacDonald, Laboratory of Applied Dermatopathology (Guy's Campus) and Department of Histopathology, UMDS ([1]St Thomas and [2]Guy's Campus), London, England.

Studies indicate a pro-inflammatory role for the activated T-cell derived cytokine, IFN-γ, in cutaneous immunopathology. To determine whether IFN-γ also contributes to keratinocyte hyperproliferation in vivo, normal human volunteers received intradermal injections of recombinant human IFN-γ ($30\mu g$) and keratinocyte proliferation assessed on whole skin biopsies using 3 independent histochemical parameters; (a) silver-nucleolar organiser region (Ag-NOR) counts, (b) proliferating cell nuclear antigen (PCNA), and (c) hyperproliferative keratin 16 (K16) expression.

Results obtained from biopsies taken on day 6 following first IFN-γ injection revealed significantly increased scores for each parameter. (a) AgNOR count per nucleus: IFN-γ mean = 3.99 (sem = 0.2), control = 2.48 (sem = 0.42)($p<0.01$), (b) PCNA (per hpf); IFN-γ = 40.9 (sem = 3.5), control = 16.6 d(sem = 3.5) $p<0.81$, and (c) K16; IFN-γ = 1 07 (sem = 0.36), control = 0.04 ($p<0$ 01). Proliferation indices were not elevated at 48 hours following IFN-γ injection. As expected proliferation indices were upregulated in psoriatic epidermis.

These studies demonstrate that, in contrast to its in vitro growth inhibitory effects, IFN-γ induces keratinocyte hyperproliferation in vivo, indicating both a pro-inflammatory and growth regulatory role for this cytokine. Since IFN-γ production is detectable in psoriatic epidermis, these findings may be of particular importance to the pathophysiology of this disease.

DO HUMAN ORAL AND EPIDERMAL KERATINOCYTES MAINTAIN THEIR PHENOTYPE *IN VITRO* ?

L.Selvaratnam, A.T.Cruchley,[1] H Navsaria, [1] I.M Leigh, D M Williams.

Depts. of Oral Pathology & [1]Expt Dermatology, London Hospital Medical College, London.

Oral mucosa and skin diverge markedly in their phenotype as stratified squamous epithelia, although both serve a common protective function and are derived from embryonic ectoderm The role of mesenchyme in influencing epithelial differentiation and maintaining site-associated phenotype in the adult is not clearly defined.

To address this issue, we investigated the extent to which oral and epidermal keratinocytes retained their phenotype of origin when cultured in the absence of their supporting connective tissue. Keratinocytes harvested from human buccal mucosa and trunk skin were seeded at passage 2 & 3 onto inert collagen membranes (Cellagen Discs, ICN Biomedicals) and grown for 10 days as either submerged or interface cultures (n=5 each) in supplemented DMEM/F12 without feeders. Cultured epithelial sheets and biopsies of normal buccal mucosa and skin were then processed for ultrastructural comparison.

All cultures stratified into 5-10 cell layers and exhibited features of differentiation in common with their *in vivo* counterparts. The buccal keratinocyte cultures had similar cell morphology, tonofilament organisation and membrane-coating granule (MCG) structure to normal buccal mucosa, but the numbers of cell layers, cellular interdigitations and desmosomes were all decreased in culture. Cultured epidermal keratinocytes had similar numbers of cell layers, similar tonofibril organisation, desmosomes and keratohyalin granules to intact skin but cornification, only seen in interface cultures, was patchy. Such markers of epithelial maturation as MCGs were seen to approach more normal morphology in interface cultures of both buccal mucosa and skin. Lipid inclusions peculiar to cultures were also evident.

It is concluded that human buccal and epidermal keratinocytes obtained from adults express phenotypic characteristics which correspond to their site of origin to varying degrees, in the absence of mesenchymal influences.

Ms L.Selvaratnam is in receipt of a Unilever Research Scholarship

A STUDY OF HUMAN PAPILLOMAVIRUS (HPV) IN CUTANEOUS TUMOURS FROM RENAL ALLOGRAFT RECIPIENTS (RAR). L.A. Stark, M.J. Arends, H. Shahidullah*, K.M. McLaren, E C. Benton*, J.A.A. Hunter* & C.C. Bird. Departments of Pathology and *Dermatology, University of Edinburgh.

It is now well established that renal allograft recipients (RAR) have a higher incidence of skin cancer than the general population. Several factors have been implicated in the development of these tumours including infection with HPV. Multiple skin biopsies were obtained from over 40 RAR from the south-east of Scotland. These were screened for the presence of HPV DNA using type specific PCR for HPV 1,2,5 and 8 with primers located in E6, and by Southern analysis using mixed probes for common cutaneous and EV types. Lesions were divided into four histological categories and results were compared with those from perilesional and uninvolved skin from the same patients. Preliminary results show 44% of squamous cell carcinomas, 37% of intra-epidermal carcinomas, 53% of keratoses (verrucous and actinic) and 77% of typical viral warts contained HPV DNA.

HPV types 1,2 and 5, but not 8, were detected by PCR. However, more lesions were positive for HPV by Southern analysis suggesting that types other than HPV 1,2, 5 and 8 were present in many cases. A small subgroup of patients had multiple tumours of differing HPV status. These data suggest that HPV may be one of the factors contributing to skin carcinogenesis in RAR.

AGGREGATION BEHAVIOUR OF HAIR FOLLICLE DERMAL CELLS IN VITRO M Sleeman and C Jahoda; Univ of Durham, Dept of Biol Sciences, Science Laboratories, South Road, Durham, DH1 3LE, U K.

Specialised fibroblasts from the dermal component of adult vibrissa follicles, dermal papilla (DP) and dermal sheath (DS), reveal aggregative properties in vitro that are distinct from skin fibroblasts Two aspects of this phenomenon - the causal factors of clump formation, and the stability of cell aggregates once formed - have been investigated in vitro by cell mixing experiments, physical wounding, and migration assays using microwell chemotactic chambers In repeat experiments which involved twelve replicates for each sample, minimal essential medium conditioned by DP cells during aggregate formation elicited a chemotactic response from flank skin fibroblasts that was significantly greater than that to either 10% fetal bovine serum, or conditioned medium from established DP cell aggregates Evidence that cells from highly clumped cultures are movement inhibited comes from wounding experiments on four different DP cell lines Aggregate cultures scored with a pipette tip "repaired" uncovered substrates slowly, and did not form new clumps in the wound spaces Autoradiography on 2 papilla cell lines mixed with paired skin fibroblasts (where one cell type was prelabelled with tritiated thymidine) showed that DP cells "sort out" and form similar sized and patterned aggregations regardless of their relative numbers as compared to fibroblasts. Aggregation is vital for the development and maintenance of hair follicles This work provides some evidence of dual mechanisms involved, that is (a) chemotactic activity in the creation of aggregates and (b) an enhanced cohesiveness of the clumps once established as a result of changes to the cells The molecular elements that underpin DP cell aggregate formation and DS cell migratory activities are the subject of current investigations

RAPID INDUCTION OF P AND E SELECTINS BY NEUROPEPTIDES IN NORMAL HUMAN SKIN

CH Smith, JNWN Barker, TH Lee*, DM MacDonald. Laboratory of Applied Dermatopathology, St John's Institute of Dermatology & Dept Allergy & Allied Respiratory Disorders*, UMDS (Guy's Campus) London.

Neuropeptides (NP) have recently been implicated in a number of inflammatory dermatoses including eczema and psoriasis Since endothelial cell (EC) adhesion molecule expression is crucial to recruitment of inflammatory cells into tissue, we have examined their expression in normal human skin following intradermal (25ul) calcitonin gene-related peptide (CGRP) (2uM), vasoactive intestinal polypeptide (VIP) (2uM), substance P (SP) (1uM) and phosphate buffered saline Biopsies were taken at times 0, 15 minutes, 1, 4 and 8 hours (n=3 for each time point), and sections examined by quantitative immunohistochemistry. CGRP, VIP and SP induced rapid translocation of P selectin (GMP-140) from dermal EC cytoplasmic Weibel-Palade granules to luminal membranes at 15 minutes and 1 hour. Marked upregulation of E selectin (ELAM) was observed at 4 and 8 hours Coincident with these changes, was a marked neutrophil infiltrate, first observed at 15 minutes and increasing up to 8 hours No change in constitutive EC ICAM-1 expression was observed following NP.

Current findings demonstrate induction of P and E selectins on dermal EC together with rapid neutrophil recruitment into skin following CGRP, VIP and SP. These NP, found in cutaneous autonomic efferent, and sensory nerve afferent fibres, may thus initiate cutaneous inflammatory events in vivo through induction of EC leukocyte adhesion molecules.

DOMINANT DYSTROPHIC EPIDERMOLYSIS BULLOSA : A GENETIC, IMMUNOFLUORESCENT AND ELECTRON MICROSCOPIC STUDY.

G Taylor, E Chow ,[1]F M Pope, F Wojnarowska , [2]P R Millard. Department of Dermatology, The Slade Hospital, Oxford, [1]Clinical Research Centre, Northwick Park Hospital, London, and [2]Department of Histopathology, John Radcliffe Hospital, Oxford.

Dystrophic epidermolysis bullosa (EB) is an inherited blistering disorder. Anchoring fibrils, consisting mainly of collagen type VII, have been shown to be abnormal in some forms of this condition.

We studied a 3 generation family with classical autosomal dominant dystrophic EB. It contained 10 affected individuals of whom 9 were available for study and one was deceased. Blood samples were obtained from 16 relevant family members and analysed with the anonymous marker D3S2 close to the collagen type VII locus. The disease clearly segregated with the plus marker but because of the distribution of markers within the family, this is relatively uninformative. It did however give a positive score because of the clinical phenotype and this could be added to the score of the other three families so far tested giving a total lod score in excess of 9.6 theta = 0. Like the other British families with larger lod scores the mutation segregates with the plus haplotype suggesting the possibility of a common mutation in all four of them.

We obtained skin biopsies from the clinically uninvolved forearm skin of 6 affected members of the above family. The biopsies were incubated with sera from a patient with epidermolysis bullosa aquisita (EBA) and monoclonal antibodies to both the carboxy and amino terminal ends of collagen VII and then visualised by standard immunofluorescent techniques. There was normal staining with collagen VII monoclonals but three patients showed equivicol staining with EBA serum which contains circulating antibodies to normal collagen type VII. Skin from two patients was examined by electron microscopy and the anchoring fibrils had a normal appearance.

In summary, no significant abnormality of collagen type VII expression was detected on indirect immunofluorescence but the genetic studies point to a possible mutation in the region of the collagen VII locus. This suggests that in this family, collagen VII is produced, but in an abnormal form.

α-MELANOCYTE-STIMULATING HORMONE INDUCES TYROSINASE mRNA, TYROSINASE ACTIVITY AND MELANOGENESIS IN B16 F1 MURINE MELANOMA CELLS.

D. Tobin, G. Hunt, J.L. Rees, J.E. Cresswell & A.J. Thody, Dermatology Department, University of Newcastle upon Tyne, UK.

Tyrosinase, which catalyses the conversion of tyrosine to dopa and subsequently dopa to dopaquinone, is essential for the production of melanin. Thus tyrosinase is likely to be an important control point in melanogenesis. α-Melanocyte-stimulating hormone (α-MSH) stimulates melanogenesis at the level of tyrosinase and has been shown to act post-translationally to activate the enzyme. In this study, we have investigated the possibility that α-MSH may also have an effect on tyrosinase mRNA levels in B16 F1 murine melanoma cells.

B16 F1 cells were cultured in the presence (10^{-10} - 10^{-8}M) or absence (control) of α-MSH for 72 hrs prior to RNA extraction. Tyrosinase mRNA was measured by Northern blot analysis of total cellular RNA and probed with a ^{32}P-labelled cDNA insert of phc TYR6. A single transcript of 2.1 Kb, corresponding to tyrosinase, was observed. Stimulation of the cells with α-MSH resulted in a dose-dependent increase in this transcript. In a parallel experiment, there was a significant correlation between tyrosinase activity (measured spectrophotometrically at 490nm with dopa as the substrate) and α-MSH dose: Spearman's rank correlation coefficient r_s = 0.92, $p<0.01$. In addition, melanin content of the cells was also related to α-MSH concentration: r_s = 0.95, $p<0.05$.

In conclusion, we have demonstrated that α-MSH stimulation of B16 F1 cells results in a dose-dependent increase in tyrosinase mRNA and concomitant increases in tyrosinase activity and melanin content. Thus, while α-MSH activates tyrosinase through a post-translational mechanism, our results suggest that the peptide may also act pre-translationally.

IMMUNOHISTOLOGICAL MARKERS FOR ARTHRITIS IN PATIENTS WITH PSORIASIS

D.Veale,L.Barnes,S.Rogers,O.FitzGerald.
The City of Dublin Skin and Cancer Hospital,Hume St.and St.Vincent's Hospital,Dublin.

An arthritis develops in 5-7% of patients with psoriasis but the reason for this is not clear. It has been suggested that the presence of HLA-DR+ keratinocytes is a marker for arthritis in patients with psoriasis(1). The aim of this study was to examine the immunohistological features in the involved skin of patients with psoriatic arthritis and to compare the changes to skin from patients with psoriasis but no arthritis and to normal skin from patients without psoriasis. Samples were stained with monoclonal antibodies against T cells (CD3,CD8,CD4,CD5Ro), B cells(CD20),macrophages (mac387), vascular endothelium(FV111-related antigen)and a Langerhans cell marker (R155). Serial sections of skin were examined in order to estimate the presence of DR+ keratinocytes.

Fifteen patients were studies, all had psoriasis vulgaris and they were rheumatoid factor negative,eight had an asymmetric oligoarthritis and seven had a symmetric polyarthritis. Results indicate a mononuclear cell infiltrate composed mainly of CD4 + CD45RO T-cells in the involved skin. There were significantly more CD45RO T-cells, B-cells and blood vessels in the involved skin of psoriatic arthritis patients compared to psoriasis and normal controls ($p<0.02$). Furthermore, there were significantly more DR+ cells in the epidermis of the involved skin in the psoriatic arthritis group when compared to the psoriasis group ($p<0.02$), however DR+ keratinocytes were present in the epidermis of both groups i.e. the psoriasis arthritis group and the psoriasis only group.

In conclusion, this study suggests that increased vascularity, increased DR+ epidermal cells and the presence of B-cells but not the presence of DR+ keratinocytes are markers for arthritis in patients with psoriasis.

References.

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CICATRICIAL PEMPHIGOID: ANALYSIS OF CIRCULATING AND BOUND ANTIBODY ISOTYPES AND C3.

V Venning, M Nayar F.Wojnarowska, J Allen and K.Schomberg Department of Dermatology, The Slade Hospital, Oxford.

Recent studies suggest that the target antigen(s) in cicatricial pemphigoid (CP) and bullous pemphigoid (BP) are identical, and that other factors must explain the clinical differences. Differences in antibody isotypes might be such a factor.

Fourteen biopsies and 31 sera from 31 CP patients were analysed. Both the biopsies and the normal human skin were studied intact and after splitting with 1M NaCl. The isotypes of the circulating and bound antibodies, the subclasses of the IgG and IgA antibodies, and C3 were demonstrated using a standard immunofluorescence technique.

% Positive

	G	G1	G2	G3	G4	A	A1	A2	M	C3
Direct										
Intact	21	7	7	7	14	0	7	0	0	21
Split	43	36	36	14	21	7	0	0	0	7
Indirect										
Intact	29	16	10	3	19	0	0	0	0	6
Split	39	16	13	6	23	19	19	0	3	13

The results are shown in the table. In the main, the use of split substrates enhanced the detection of immunoreactants. IgG and C3 were the commonest immunoreactants, and IgG1 and IgG4 the main subclassses. The presence of IgG1 and C3 did not correlate. Circulating IgA antibodies were detected more commonly than bound IgA, but only on split skin, all were IgA1. Circulating IgA antibodies were not associated with C3. There was no advantage in the use of subclasses for the detection of circulating or bound IgG and Ig A

These results do not differ markedly from our and other previous findings in BP, and suggest that factors other than antibody isotype determine disease expression.

This work was supported by an Oxford Regional Health Authority Grant

FAILURE TO DETECT REARRANGEMENTS OR LINKAGE OF THE RARβ GENE IN PSORIASIS.

I.Williamson, A.Taylor, J.L.Rees. Department of Dermatology, University of Newcastle upon Tyne.

Retinoids exert a variety of potent effects on epithelial differentiation which are thought to be mediated by a family of ligand inducible transactivating nuclear receptors. RAR-β is known to be induced in response to ligand and, given the therapeutic effects of retinoids in a variety of dyskeratoses, we wished to examine whether abnormalities or deletions of this receptor may be associated with dyskeratotic conditions such as psoriasis. High molecular weight DNA was extracted from venous blood according to standard protocols from a variety of patients with psoriasis and other disorders of keratinisation, and subjected to digestion with a panel of restriction enzymes, Southern blotted, and probed with an oligo-labelled RAR-β cDNA probe and washed up to two times SSC at 65°. We detected no polymorphisms in normal or diseased subjects ($n=40$) with a variety of 20 restriction enzymes but can confirm a previously described MspI polymorphism (Datson et al. Nuc.Acid.Res. 1989, 17(21), 8897): constant bands of 0.74KB, 1.4KB and 1.6KB and 7.1KB were detected, together with a simple polymorphic 8.1 or 7.7KB band with allele frequencies of 0.8 and 0.2 respectively in this population. We observed no RARβ rearrangements in patients with psoriasis, nor association of any particular RFLP pattern with the disease in the subjects studied. We conclude that psoriasis is unlikely to be in linkage disequilibrium with the RAR-β gene.

THE SELECTIVE INFLUX OF CD25+ LYMPHOCYTES INTO THE EPIDERMIS OF NEGATIVE AS WELL AS POSITIVE PATCH TESTS.

C.M. Willis and J.D. Wilkinson, Dept. of Dermatology, Wycombe General Hospital, High Wycombe, Bucks.

The infiltration of activated lymphocytes bearing the IL-2 receptor (CD25) into the skin has been reported to occur in a variety of dermatoses. In this study we wished to examine whether activated lymphocytes are a feature of irritant contact dermatitis and, in particular, whether the lymphocytic response varies between lesions induced by structurally unrelated chemical irritants. 48h patch test reactions to sodium lauryl sulphate, benzalkonium chloride, croton oil, nonanoic acid, propylene glycol and dithranol were biopsied and frozen sections immunolabelled with an antibody against CD25, the antibody/antigen reaction being visualized using the avidin biotin peroxidase system. Appropriate vehicle, occlusion and normal skin controls were also included. Quantitative analysis revealed an increase in the absolute number of CD25+ lymphocytes in almost all patch tests. Importantly, however, when analyzed on the basis of % of total lymphocytes that were CD25+, significant increases were present in the epidermis of the vehicle and occlusion control groups only ($p<0.05$); there being no such increase in the irritant groups relative to their appropriate controls. This indicates that patch testing per se induces physiological disturbances in the skin, possibly including the release of IL-1, sufficient to initiate the selective influx of CD25+ lymphocytes into the epidermis.

A QUANTITATIVE STUDY OF LANGERHANS CELLS IN HIV-1 POSITIVE PSORIATIC SKIN.

V. Zemelman, V. van Neer, N. Patel, J. Langtry, R.C.D. Staughton. Skin and Theurapeutics Research Laboratory, Westminster Hospital, SW1P 2AP, UK.

The association of severe psoriasis with HIV-1 infection raise interesting questions about the role of epidermal Langerhans cells (LC) in the skin of these patients. The aim of this study was to measure the number of LC in the lesional and non-lesional skin from seven HIV-1 positive psoriatic patients and compared the results with age, sex, site matched HIV-1 negative psoriatic controls.

The number of Langerhans cells were determined by staining with S100 polyclonal antibody using 3-step avidin-biotin immunoperoxidase method and positive cells were counted above basal layer and evaluated as: cells per mm ^{2} of epidermal surface and cells per mm of length of basement membrane.

Immunohistological evaluation revealed a significant reduction (P<0.005) in the number of LC per mm ^{2} epidermal surface in the lesional skin from HIV-1 positive (25.92 ± 22.7) and HIV-1 negative (64.8 ± 41.4) compared with non-lesional skin from HIV-1 positive (104.72 ± 19.7) and HIV-1 negative (151.8 ± 50.3).

This reduction in the number of LC in lesional skin in both groups of patients wa- also observed when LC were expressed per mm of length of basement membrane.

HIV-1 positive psoriatic patients show a significant decrease (P<0.005) in the number of epidermal LC in both, lesional and non-lesional skin compared with the control group HIV-1 negative psoriatic patients using both methods of counting.

These results show that the exacerbation of psoriasis observed in HIV-1 positive skin is closely associated with a marked reduction in the number of epidermal Langerhans cells.

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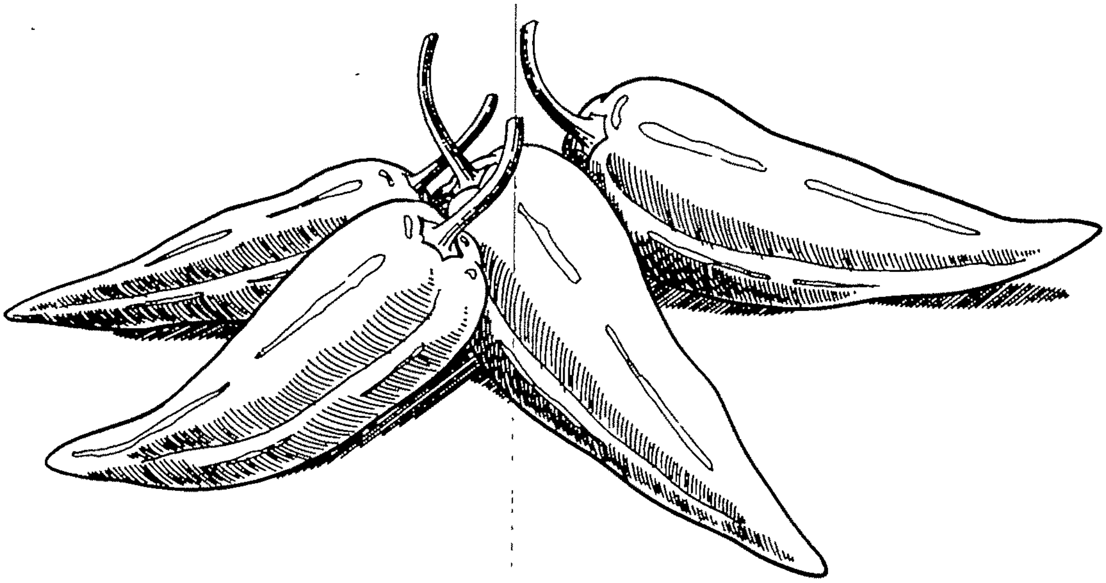

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British Journal of Dermatology (1992) **127**, 442–449.

Correspondence

A case of malignant haemangioendothelioma showing response to interleukin 2 therapy

SIR, Malignant haemangioendothelioma (MHE) is a rare tumour which, in its advanced stages, is refractory to treatment.[1] While the effectiveness of interleukin 2 (IL-2) in the treatment of MHE has yet to be established,[2,3] the following case report should provide sufficient basis to encourage further studies.

The patient was an 86-year-old woman whose management was complicated by her age and a history of coronary disease. A small nodule appeared on the right side of the patient's forehead approximately 1 year after trauma to the area. Because there was only gradual increase in the size of the nodule, she delayed seeking medical advice for 3 months. She eventually attended our clinic in August 1989. The nodule had reached a size of 65 × 53 mm, was soft in texture and dusky red in colour (Fig. 1). Several papules of the same colour were present in the area immediately surrounding the nodule. Although not ulcerated, the nodule was prone to bleed. There was no regional lymphadenopathy.

Histology of a skin biopsy showed numerous capillaries in the upper dermis lined by a single layer of large, cuboidal, pleomorphic endothelial cells (Fig. 2).

A diagnosis of MHE was made, based on the clinical appearance and histopathological findings. Because of the patient's age and heart condition, we decided against surgical treatment, opting instead for IL-2 therapy. Initially, the temporal artery which fed the tumour was used as a route for IL-2 injection by pump, at a rate of 2000 units/day for a period of 7 days. This was followed by a series of subcutaneous and local injections in order to avoid bleeding and infection. By this means, 2000 units/day were administered 3 days/week for a period of 31 weeks. By April 1990, after 8 months of continuous treatment totalling 200,000 units of IL-2, the tumour showed considerable reduction in size, and the formation of scar tissue was observed (Fig. 3). However, by August 1990, with the total dose of IL-2 having reached 300,000 units, a resumption of bleeding and the appearance of new nodules occurred, even at the local injection sites. Histology of the new nodules showed the same features as the original tumour. Throughout the entire treatment period, no systemic adverse effects were observed.

IL-2 is said to activate CD4 (helper) and CD8 (suppressor) lymphocytes[4] or CD16 (natural killer) cells[5] through cortisol or endorphin secretion.[6] Comparison of biopsy specimens obtained from the original nodule, and nodules that later appeared around IL-2 injection sites, showed considerable similarity in appearance. The infiltrating cells were predominantly T cells, but there was no evidence of an increase of CD4, CD8 or CD5-positive cells by immunochemical staining such as CD71 or HLA-DR.

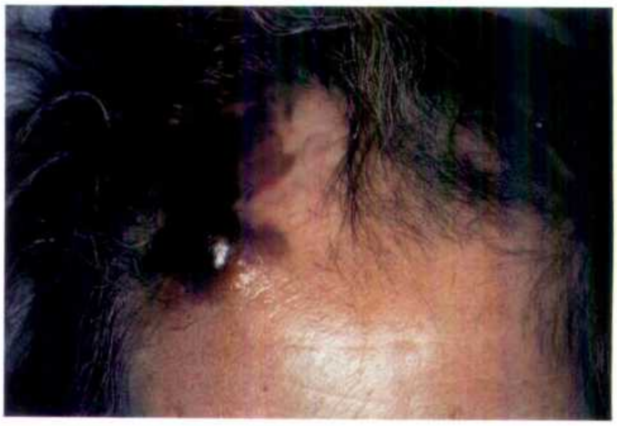
Figure 1. Initial appearance of the tumour.

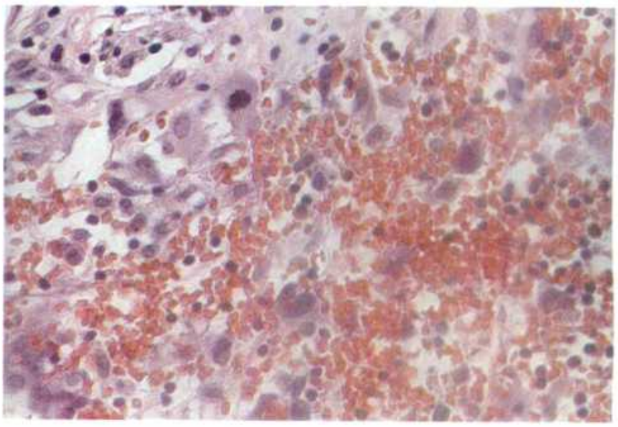
Figure 2. Histology showing capillaries of various sizes in the upper dermis. The capillaries are lined by large, cuboidal, pleomorphic endothelial cells.

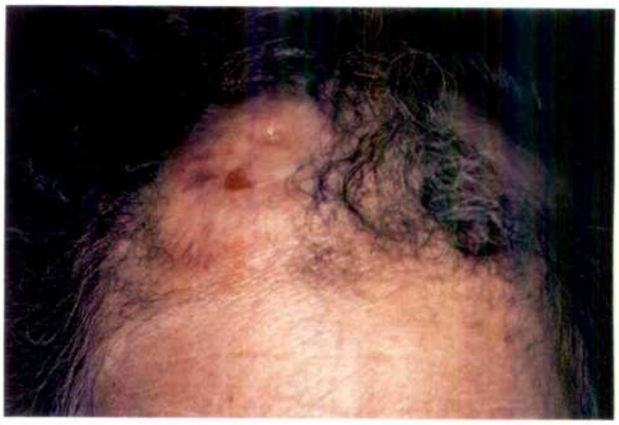
Figure 3. Tumour regression after injection of 200,000 units of IL-2.

The suppressive factors which some authors suggest are secreted by tumour cells[7,8] may have impaired the response to IL-2. As the histological findings indicated no reaction to IL-2, it is possible that the dose was too low in relation to these suppressive factors to activate lymphocytes or natural killer cells. It is reasonable to assume that a higher dose of IL-2 might have caused complete necrosis of the MHE tissue by activation of the immune system. However, as pointed out by Whitehead *et al.*,[9] a higher dose of IL-2 could conceivably produce toxic side-effects.

MHE is more common in the elderly, who may be poor surgical risks. IL-2 therapy is considered an attractive option for such patients, because no other acceptable treatment currently exists, and significant, albeit temporary, remission was observed in our patient.

We conclude that IL-2 therapy for MHE demonstrates encouraging possibilities for patients who are poor surgical risks. Although remission was temporary in this case, the response supports the potential of IL-2 treatment. However, since detailed treatment protocols have not yet been established, further studies are strongly recommended.

Department of Dermatology,
Surugadai Hospital, Nihon University,
1–8–13, Kanda Surugadai, Chiyoda-ku,
Tokyo, Japan

T.INADOMI
A.FUJIOKA
H.SUZUKI

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Subacute cutaneous lupus erythematosus-like gyrate erythema and hypertrichosis lanuginosa acquisita associated with uterine adenocarcinoma

SIR, We report a patient who presented with a subacute cutaneous lupus erythematosus-like gyrate erythema and hypertrichosis lanuginosa acquisita—both recognized paraneoplastic markers. The patient was subsequently found to have a uterine adenocarcinoma.

A 65-year-old woman presented with a 6-month history of a generalized rash associated with weight loss, fatigue and the development of excessive facial hair. There was no significant past medical history, and no previous history of cutaneous disease. She was not taking any medication.

On examination the patient was thin and clinically anaemic. There was no lymphadenopathy. Hypertrichosis was present affecting her face, ears and neck. There were no signs of virilization. A gyrate erythematous rash with peripheral scaling was present on her trunk, buttocks and limbs, with sparing of the face. Physical examination was otherwise normal.

Investigations revealed a haemoglobin of 8·6 g/dl, normocytic indices, and normal white cell and platelet counts. The ESR was 150 mm/h. Liver function tests: alkaline phosphatase 1699 U/l (90–280 U/l), γ-glutamyltransferase 436 U/l (5–60 u/l). Biopsy of involved skin showed histological features consistent with subacute lupus erythematosus. However, direct immunofluorescence microscopy of both involved and uninvolved skin showed no immunoglobulin or complement deposition. Screening for autoantibodies, including anti-Ro and anti-La was negative. Serum immune complexes were not detected, and complement levels were normal. Chest X-ray was normal. Ultrasound examination identified a left-sided pelvic lesion with associated free fluid. Examination under anaesthetic revealed an enlarged, bulky uterus with a fixed, left-sided irregular pelvic mass. Uterine curettings confirmed the presence of a uterine adenocarcinoma. Because of the patient's poor general condition and stage of disease, medroxyprogesterone hormonal therapy was commenced. The skin lesions improved over a period of several weeks, but the hypertrichosis remained unchanged.

Variants of cutaneous lupus erythematosus (CLE) have been recognized to be associated with underlying neoplasia. These include subacute cutaneous lupus erythematosus,[1,2] lupus erythematosus gyratus repens[3,4] and subacute lupus erythematosus-like gyrate erythema.[5] Subacute cutaneous lupus erythematosus (SCLE) is a distinct form of CLE associated with a non-scarring, papulosquamous or annular rash,[6] with characteristic immunological and immunogenetic abnormalities.[7] In our patient the rash differed from the characteristic form of SCLE in that there was a lack of photosensitive distribution, with sparing of the face, and involvement of covered sites. There was also absence of immunoglobulin deposition at the dermo-epidermal junction. Immunoglobulin deposition is present in 60% of cases of SCLE.[6] Antinuclear factor, anti-Ro and anti-La antibodies are present in 63, 60 and 15% of SCLE cases, respectively, [6,7,8] but in our case these were negative.

Hypertrichosis lanuginosa acquisita is the acquired development of colourless or pale, fine hairs of variable extent over the body, and is well recognized as a cutaneous marker of internal malignancy.[9] The most common associated neoplasms are carcinoma of the lung and carcinoma of the colon.[10] Hypertrichosis lanuginosa acquisita has been reported in association with adenocarcinoma of the uterus on two previous occasions.[11,12]

This is the first report of a patient with uterine adenocarcinoma heralded by a combination of these two paraneoplastic cutaneous conditions. This emphasizes the need to screen patients presenting with these signs for the presence of internal malignancy.

*Department of Dermatology and *Pathology,*
Belfast City Hospital,
Belfast BT9 7AB,
Northern Ireland.

K.E.McKenna
D.Hayes*
J.C.McMillan

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Unusual pattern of phototoxic burning following trimethylpsoralen (TMP) bath photochemotherapy (PUVA)

Sir, Trimethylpsoralen (TMP) bath PUVA is an established psoriasis treatment used particularly in Scandinavia but also in mainland Europe. It has been attracting increasing attention for its advantages in selected patients. We would like to draw attention to an adverse-effect of this treatment which, to our knowledge, has not been reported previously.

Shortly after we introduced TMP bath PUVA, three patients in the early stages of their treatment developed unusual patterns of phototoxic burning which necessitated modification of the treatment.

The first, a 25-year-old woman, developed a painful linear erythema of her chin and neck (Fig. 1) 48 h after UVA irradiation. Subsequent enquiry revealed that this line corresponded to the bath water level. The second patient, a 21-year-old woman, developed phototoxic burning at sites in contact with the bath: on the back of her shoulders and arms (Fig. 2) and buttocks. The third patient, a 47-year-old male, developed a linear phototoxic burn affecting the backs of his shoulders and backs of his arms, again corresponding to the bath water level. It transpired that, contrary to our advice, he was a bathtime reader!

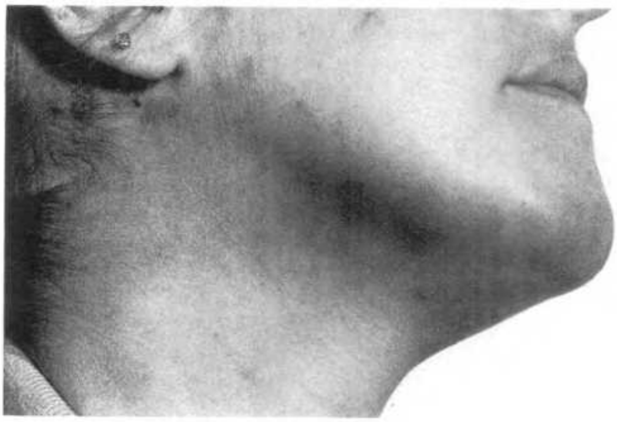

Figure 1.

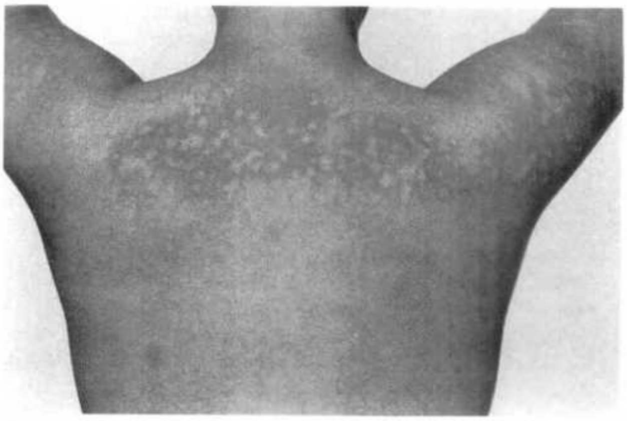

Figure 2.

TMP powder appears to be virtually insoluble in water. HPLC studies indicate variable concentration in the bath. Furthermore, filtered TMP bath water contains virtually none of the drug (unpublished data generated from our ongoing studies into TMP solubility). It appears that despite attempts to dissolve the powder in boiling water, it remains a microcrystalline suspension within the bath. It is therefore likely that the varying distribution within the bath water results in a variation in the pattern of cutaneous phototoxicity. We are at present investigating different ways of dissolving/suspending the TMP powder in bath water.

In the meantime, we have overcome our patients' problems by advising them to agitate the water continually with their hands during immersion in the bath. In addition, they are also asked to vary intermittently their position in the bath. This appears to produce a more uniform drug distribution and prevent the unusual patterns of phototoxicity experienced by our first few patients.

We continue to use TMP bath PUVA for selected patients. It has particular advantages over systemic PUVA in the treatment of patients who have renal or hepatic failure or, (in view of its higher photosensitizing capacity and lower UVA irradiation times), those who are unable to tolerate long treatment times.

Photobiology Unit,
Ninewells Hospital,
Dundee DD1 9SY, U.K.

S.A.George
J.Ferguson

Lesional blistering following narrow-band (TL-01) UVB phototherapy for psoriasis: a report of four cases

Sir, Narrow-band (312±2 nm) UVB phototherapy, using Philips TL-01 fluorescent lamps, is being increasingly used in the management of psoriasis, primarily because of its reported increased efficacy and safety when compared with older broadband UVB (270–350 nm) sources.[1] Over the past 6 years our experience of treating 800 patients has revealed an uncommon blistering problem, which develops towards the middle of a treatment course, and occurs at the site of psoriatic lesions. We wish to outline our experience of four cases who have developed this problem.

Our routine TL-01 phototherapy programme involves commencing treatment at 70% of the patient's minimal erythema dose (MED) value and thereafter thrice weekly irradiation, with increments of 40%, to a maximum dose of 2000 mJ/cm² (increments being reduced when adverse effects occur). Therapy is conducted in a stand-up cubicle with 50 tubes and a mean irradiance of 3·8 mW/cm².

Case 1. An 18-year-old woman, skin type 1, with a 2-year history of psoriasis refractory to topical therapy, developed asymptomatic blisters at the sites of virtually resolved psoriatic plaques on her abdomen and breasts during narrow-band UVB (TL-01) phototherapy. The blisters varied in size from 0·5 to 1·5 cm in diameter. Blistering arose 12–24 h after her fifteenth treatment at 1074 mJ/cm², representing 3·8 times her initial MED. No systemic or topical photosensitizers had been used; psoriasis, had previously received four courses of oral 8-MOP PUVA and two courses of TL-01 phototherapy with good results, and no adverse effects. During her third course of her only medication was the oral contraceptive pill. Apart from the mild erythema seen routinely in such patients, her course prior to the eruption of bullae had been unremarkable. The blisters resolved spontaneously despite continuation of treatment without further dose increments. Routine investigations, including plasma porphyrin screen and ANA, were negative. Histology of a skin biopsy demonstrated a blister containing fibrin and neutrophils, with a roof of necrotic keratinocytes. The underlying epithelium lacked a granular layer and was mitotically active. A mild perivascular inflammatory infiltrate was present in the dermis. The appearances suggested a re-epithelializing subepidermal blister. Direct immunofluoresence was negative.

Case 2. A 15-year-old girl, skin type 3, developed asymptomatic blistering (Fig. 1) at the sites of resolving psoriasis after 11 irradiation sessions at a dose of 1289 mJ/cm², representing 3·3 times her initial MED. Uninvolved skin did not show abnormal photosensitivity. Her irradiation dose was reduced, and subsequent increments were restricted to 10% of her previous dose. Her subsequent treatment was uneventful despite receiving doses higher than that which presumably induced the blisters. She was not receiving either topical or systemic photoactive therapy.

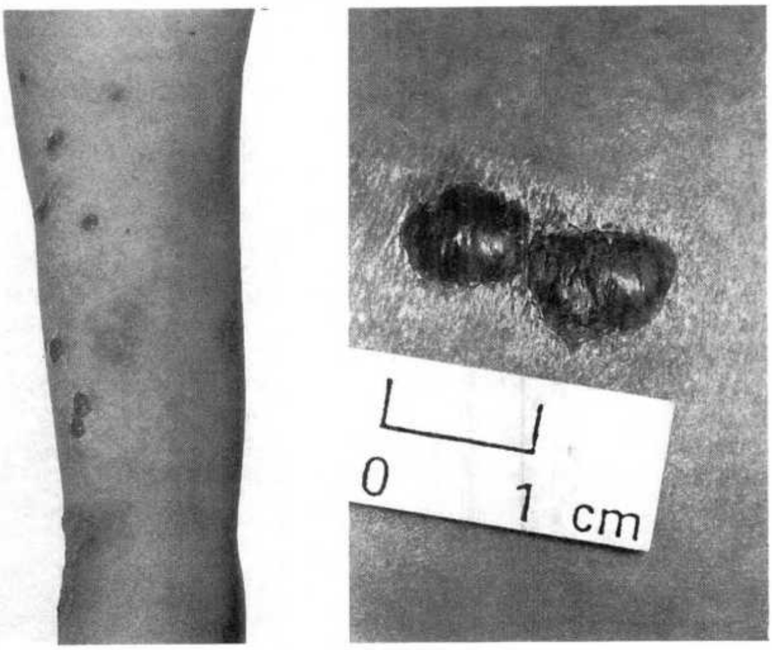

Figure 1. Case 2. Blisters occurring on resolving plaques of psoriasis.

Case 3. A 21-year-old woman, skin type 2, with long-standing psoriasis, had previously received four courses of oral 8-MOP PUVA and two courses of TL-01 phototherapy with good results, and no adverse effects. During her third course of narrow-band UVB (TL-01) treatment she developed painful blistering affecting psoriatic plaques on her shoulders, 24 h after her twenty-third treatment at an irradiation dose of 1856 mJ/cm^2, representing 2·4 times her initial MED. The blisters healed in 2 days and treatment was resumed using our routine protocol, with complete clearance of her psoriasis.

Case 4. A 44-year-old male psoriatic, skin type 2, receiving his first course of narrow-band UVB phototherapy, developed painful blistering on psoriatic plaques in the interscapular area and the backs of his shoulders. This occurred after his seventeenth treatment session at a dose of 1745 mJ/cm^2, which was 4·5 times his initial MED. Routine haematological and biochemical parameters were normal, as were plasma and urine porphyrin analyses. Antinuclear antibody was negative. A skin biopsy showed a large blister containing fibrin and occasional neutrophil polymorphs. The roof of the blister was formed by necrotic keratinocytes. In places, the lack of an underlying epithelium suggested a subepidermal split. Elsewhere, there was underlying epithelium lacking a granular layer, probably indicating re-epithelialization. There was a perivascular lymphocytic inflammatory infiltrate in the superficial dermis. Immunofluorescence was negative. Interestingly, this patient had previously had a course of PUVA and had developed pseudoporphyria on the dorsa of his feet in the later stages of his treatment.

Blistering during PUVA therapy following mild trauma (pseudoporphyria) is well described. Our cases do not conform to this pattern because only lesional skin was affected. Similarly, the lack of significant erythema and the unusual distribution of the lesions differentiates them from the acute blistering that may result from a generalized overdose of UV exposure.

This blistering phenomenon, which in our experience is not a feature of broad-band UVB (Philips TL-12) phototherapy, has no obvious explanation. It is possible that plaques of psoriasis, during a course of treatment with narrow-band phototherapy, have not gained the photoprotection level of surrounding normal skin. The treatment efficacy of this new UVB source may result in a loss of photoprotection by causing a rapid loss of plaque scales at involved sites, thus exposing them to what, for these areas, is a burning dose. The onset of blistering in all our patients during the second half of their treatment supports this hypothesis. It is probable that a gentler incremental irradiation regimen would reduce the risk of lesional blistering, but this must be balanced against a possible decrease in clearance rates. We are now investigating the therapeutic efficacy of less aggressive irradiation regimens than that which we are currently using.

This is our first experience of lesional blistering during psoriasis phototherapy and leads us to question whether this may be unique to narrow-band (TL-Q1) UVB treatment. It is possible that the incidence of this phenomenon is higher, as two of the four reported cases were asymptomatic incidental findings.

Photobiology Unit, S.A.George
Ninewells Hospital, J.Ferguson
Dundee DD1 9SY, U.K.

Reference

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Discoid lupus erythematosus-like lesions in carriers of X-linked chronic granulomatous disease

Sir, J.H.Sillevis Smitt *et al.*[1] found discoid lupus erythematosus-like skin lesions with atypical histological findings and negative direct immunofluorescence (DIF) in five of 15 carriers of the X-linked cytochrome-b-558-negative variant of chronic granulomatous disease (CGD). In contrast, we have seen a carrier of CGD with a centrofacial skin eruption resembling perioral dermatitis, thus clinically atypical for discoid lupus erythematosus (DLE), whereas histology and DIF were typical of DLE.

Our patient, a 29-year-old woman, has a 5-year-old son with CGD and is a proven carrier of the disease. Recurrent facial rashes after sun exposure began at the age of 13, at first affecting only the cheeks, but later also the forehead and chin. On examination a centrofacial erythema, consisting of confluent maculo-papules sparing the immediate perioral area, was present, producing a picture suggestive of perioral dermatitis. She was otherwise well.

Histology was consistent with lupus erythematosus, showing an atrophic epidermis with focal hydropic degeneration of the basal layer. In the dermis there was a patchy lymphocytic infiltrate around dilated blood vessels and hair follicles. Direct immunofluorescence showed granular IgG, IgM and IgA deposits at the basement membrane zone. Tests for antinuclear antibodies, anti-native DNA, anti-SM antigen and rheumatoid factor were negative. Serum protein electrophoresis and urinalysis were normal. Sensitivity to ultraviolet light (short and long wavelength) was normal. The nitroblue–tetrazolium slide test showed 28% positive cells (normal range 98–100%),[2] and superoxide anion production after granulocyte stimulation by phorbol myrestate acetate was 2·00 nmol O_2/million granulocytes/min (normal range $10{\cdot}52\pm1{\cdot}83$ nmol/million/min).[2] Cytochrome b determined by dithionate difference spectroscopy was 1·62 pmol/million granulocytes (normal range $6{\cdot}4\pm0{\cdot}79$ pmol/million).[2] The facial eruption cleared within 3 weeks on treatment with chloroquine 250 mg daily, sun protection and a mild topical steroid (clobetasone-17-butyrate 0·05%).

Brandrup *et al.*[3] studied skin and oral mucosa in an unselected series of 9 CGD-carriers. They described deep rosacea-like infiltrates of the face, resembling LE profundus. In addition, changes similar to chilblain LE were present on the fingers and toes. The histological changes ranged from almost pure lymphocytic infiltration of the Jessner–Kanof type to features consistent with LE, and changes similar to chronic dermatitis. This variability in histological changes is demonstrated by the finding of an appearance consistent with LE in our case, whereas in the cases of Sillevis Smitt *et al.*[1] histological findings generally did not show all the elements typical of LE.

The pathogenesis of LE-like manifestations in carriers of CGD is unknown. By immunophenotyping of dermal infiltrates in CGD carriers, Sillevis Smitt *et al.*[1] showed that these infiltrates did not differ from those in common DLE. This suggests that the pathogenesis is the same. In CGD, ineffective phagocytosis might lead to chronic antigenaemia, eventually provoking autoantibody formation. On the other hand, negative autoimmunological findings in most cases are an important argument against this theory.[4,5]

Other common mucocutaneous disorders in carriers of CGD are recurrent aphthous stomatitis (RAS)[3,5,6] and hidradenitis suppurativa.[3,4] In contrast with common RAS, ulcers occur predominantly on the gingivae, show a more extensive erythematous halo, and have negative DIF.

Prenatal diagnosis is available for pregnancies at risk of CGD. In order to establish an effective procedure to detect and counsel carriers, women with DLE in combination with recurrent aphthous stomatitis, hidradenitis suppurativa, or a family history of early childhood deaths should be screened.[4]

Department of Dermatology,
University Hospital of Zurich,
8091 Zurich, Switzerland

J.HAFNER
A.ENDERLIN
R.A.SEGER*
B.WÜTHRICH
L.BRUCKNER-TUDERMANN*
P.PANIZZONI
G.BURG

**Children's Hospital,*
Division of Immunology and Haematology,
University of Zurich,
Steinwiesstr 75,
8032 Zurich, Switzerland.

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Median nail dystrophy associated with isotretinoin therapy

SIR, Aromatic retinoids are known to cause various dystrophic nail changes. These include splitting, softening, shedding of the nail and chronic paronychia.[1] Whilst chronic paronychia is seen during therapy with isotretinoin the other changes are encountered more frequently in association with etretinate therapy.

We report the case of a 38-year-old woman who developed a median canaliform dystrophy of a thumb nail, a change not previously reported following treatment with either etretinate or isotretinoin. The patient had severe nodulocystic acne which required three separate 4-month courses of isotretinoin (1 mg/kg/day). The time intervals between each course varied from 9 to 12 months. Six weeks after commencing each of the courses of isotretinoin she developed an obvious central ridge and proximal splitting. On both sides of the central defect there were horizontal ridges, although these were more prominent on one aspect and at the proximal end, where a fir-tree configuration could be seen (Fig. 1). These dystrophic changes subsequently resolved approximately 4 weeks after the isotretinoin was discontinued. The patient had no previous personal or family history of nail abnormalities.

Whilst we accept that median canaliform dystrophy might occur by chance in a patient taking isotretinoin, the temporal relationship to the multiple courses strongly implicates isotretinoin as the causative agent. The aetiology of median canaliform dystrophy is unknown but it is typically seen in one or more nails, the thumb-nail being most commonly

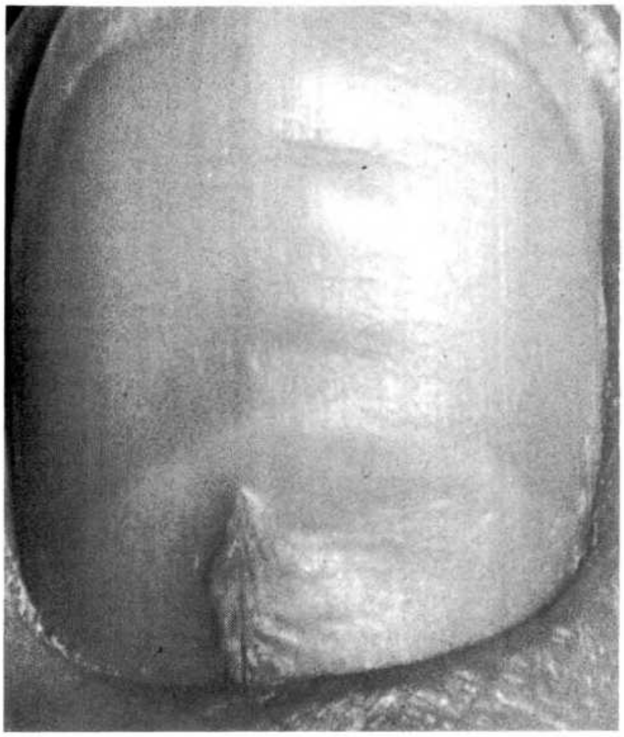

Figure 1. Median nail dystrophy, with prominent proximal splitting and ridging.

involved. It has a tendency to spontaneous remission and can occasionally be familial. In this case the cause of the dystrophy may be a dyskeratotic reaction of the keratinocytes in a localized area of the nail matrix induced by isotretinoin in a predisposed individual. We are not aware of any recorded cases of a drug-induced median canaliform dystrophy.

Department of Dermatology,
The General Infirmary at Leeds,
Great George Street,
Leeds, U.K.

W.W.Bottomley
W.J.Cunliffe

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Extracorporeal photochemotherapy in the treatment of severe psoriatic arthropathy

Sir, The efficacy of extracorporeal photochemotherapy (EP) in psoriasis and psoriatic arthropathy was first evaluated by Wilfert *et al.*[1] At present, the value of this treatment is questionable. We would like to add our experience of the use of EP in the management of one patient with long-standing psoriatic arthropathy, resistant to conventional treatment.

A 29-year-old man had a 13-year history of psoriasis and psoriatic polyarthritis. His skin had been treated with topical steroids, short-contact anthralin therapy, PUVA, etretinate, methotrexate (MTX) and cyclosporin A, following established therapeutic schedules. Arthritic symptoms improved only with oral phenylbutazone (PBZ). Although there was a marked decrease in PASI score (from 65·0 to 14·7), his arthropathy remained active, with pain, morning stiffness, disabling weakness and significant radiographic changes. In February 1991, while he was receiving treatment with weekly MTX and daily PBZ, the patient was included in an EP programme (treatment on 2 consecutive days, with a treatment-free period of 4 weeks, using a Therakos UVAR System). In August 1991, he showed a decrease in PASI score (from 14·7 to 1·0) and marked reduction in pain and morning stiffness, resulting in adequate mobility.

Based on animal models,[3] the efficacy of EP on inflammatory and autoimmune diseases is at present being evaluated, with encouraging results.[1,2,4] After 6 months treatment with EP, our patient showed a moderate improvement of both skin lesions and arthropathy. During this period, the oral MTX dose was gradually reduced from 15 mg weekly to 10 mg every 4 weeks, and at the end of the treatment period, the initial PBZ dose of 200 mg daily was reduced to 200 mg on alternate days. No adverse effects were observed. Our data agree with those of Wilfert *et al.*[1] and Vonderheid *et al.*[2] Thus, EP could be a safe and effective alternative for the treatment of refractory cases of psoriatic arthropathy. However, further objective studies are necessary to establish the real efficacy of such an expensive and time-consuming procedure.

Department of Dermatology,
Universidad de Alcalá de Henares,
Hospital Ramón y Cajal,
Apartado 37,
28034–Madrid,
Spain

R.F.de Misa
J.M.Azaña
A.Harto
A.Ledo

Correspondence:
Dr A.Ledo,
Paseo de la Castellana 167,
28046–Madrid,
Spain

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Malignant melanoma occurring as a second cancer following primary germ cell tumour in males: report of three cases

Sir, We report three male patients with malignant melanoma which developed following a primary germ cell tumour. None of the patients had the familial cancer syndrome or the familial atypical mole/melanoma syndrome. The occurrence of a second malignancy in cancer patients is well known, and it is important for those involved in their management to be alert to this possibility.

The occurrence of a malignant tumour is distressing for any affected individual. Despite the reassurance of effective therapy now available for many primary malignancies, resulting in long-term survival, it is now well known that such an individual faces an increased risk of developing a second malignancy. The following three cases illustrate the development of malignant melanoma after the occurrence of a primary germ cell tumour (seminoma or teratoma) in young adult males.

Case 1. A 26-year-old man presented with right testicular swelling in March 1986. He was found to have stage 1 seminoma, for which a right orchidectomy was performed. In February 1987, he noticed gradual enlargement of a pigmented lesion over his left scapula. Excision biopsy of the 1·6 × 1·6 cm lesion showed a malignant melanoma 0·8 mm thick, with invasion of the papillary dermis (Clark level 2). Following this, wide excision with primary closure was undertaken. There was no family history of dysplastic naevi or melanoma, nor past history of severe sunburn. His skin burned rather than tanned. A clinically 'atypical' mole removed from his left thigh in February 1988 proved benign, with no dysplastic features on histological examination. Since then the patient has remained well. Four atypical looking naevi on his trunk and left popliteal fossa have been closely observed.

Case 2. A 25-year-old man presented in March 1982 with a 3-month history of lethargy, dry cough and dyspnoea. Chest X-ray and thoracic CT-scan revealed a mediastinal mass of the left hemithorax. Biopsy at thoracotomy showed an α-fetoprotein-producing malignant teratoma (undifferentiated). Superior vena caval obstruction and cardiac failure soon ensued, necessitating intensive-care management. Diuretics and the first of an eventual six courses of chemotherapy (with bleomycin, etopside and *cis*-platinum) were given. In April 1983, a pigmented lesion was excised from his right ankle and a split-skin graft applied. Histologically, it proved to be a malignant melanoma 2·5 mm thick with invasion into and expansion of the papillary dermis (Clark level 3). There was no family history of atypical naevi or melanoma and no past history of severe sunburn. Regular review since than has revealed only one 'atypical' mole over his right scapula which has remained unchanged.

Case 3. A 26-year-old man presented in March 1983 with right testicular swelling. He had a right orchidopexy at the age of 12. Stage 1 seminoma was confirmed and a right orchidectomy was performed. In April 1988 he noticed pruritus and slight colour variation in a long-standing pigmented lesion over his right hip. Excision biopsy showed a malignant melanoma 0·45 mm thick with invasion into the papillary dermis (Clark level 2). There was no family history of dysplastic naevi or melanoma, or history of sunburn. His skin tanned rather than burned. The patient has remained well under regular review.

Survivors of primary germ cell tumours in males have been reported to have 30% more cancers than would have been predicted in the age-specific population to which they belong.[1] The most common second cancers registered were cancers of the testis, rectum and connective tissue, as well as malignant melanoma, non-Hodgkin's lymphoma and acute leukaemia. In that study (which incorporated 11 population-based cancer registries in the period 1945–1984), 16 (vs. 8·9 expected) cases of malignant melanoma were seen amongst the 17, 730 registered cases of testicular cancers. This gave a relative risk of 1·8 between the study cohort and the general population ($P<0{\cdot}05$: two-sided). Six of the 16 cases developed the melanoma in the first 4 years after their testicular cancer. Two of our three cases fall within this time category.

The Thames Cancer Registry (Belmont, Sutton, Surrey, U.K., personal communication) in the United Kingdom (covering the South-East and South-West Thames regions from 1960 to 1984, and all four Thames regions from 1985) shows four cases (vs. two expected) of malignant melanoma among 197 second primaries following testicular cancer.

It is not clear why an increased relative risk exists. However, possible explanations include the following: (i) it may be that the first cancer may predispose to the second cancer; (ii) the aetiological factor(s) responsible for the first cancer may increase the risk for the second. It has been observed, for example, that both testicular cancers[2] and malignant melanoma[3] are epidemiologically linked to higher socio-economic status; (iii) the increased medical surveillance associated with follow-up of cases with a first cancer may result in improved detection rates of second cancers; and (iv) the therapy for the first cancer may enhance the development of the second cancer. Whatever the reason(s), it behoves clinicians involved in the management and follow-up of cancer patients to be alert to the possible development of a second cancer in such patients.

Acknowledgment

We wish to thank Professor A.Horwich for permission to report these cases; Dr Martin Cook for kindly reviewing the histological specimens; and Karen Unklater of the Thames Cancer Registry for their help.

Departments of Dermatology, H.C.Chua
St George's and Royal Marsden Hospitals, P.S.Mortimer
London, U.K.

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British Journal of Dermatology (1992) 127, 450.

Book Reviews

Recent Advances in Dermatology, Volume 9 EDITED BY R.H.CHAMPION AND R.J.PYE (1992). Edinburgh: Churchill Livingstone. Pp. 218. ISBN 0-443-04451-1. Price £34.95.

This volume in the Advances in Dermatology series contains 13 topics. Although the introduction begins with the statement that 'advances in dermatology continue apace', some of the chosen topics such as 'Panniculitis: it pathogenesis and management' show depressingly little advance.

The 13 topics are predominantly clinical, and include rheumatoid arthritis and the skin, malignant lymphoma, photopheresis, tropical ulcer, and Kawasaki disease. A number of them, such as the problem of food additives in dermatology, have been fairly extensively covered in recent similar publications. These is no doubt that, with the introduction of larger numbers of monthly journals, some of which carry excellent review articles, the function of books such as those in the Recent Advances series has become a little more questionable. It is probably wise for the editors of such volumes to restrict themselves to areas that are not advancing with great speed, as these will almost inevitably be out of date by the time of publication, but look carefully for areas which have been relatively under-reported at the present time.

I personally found the chapter on malignant cutaneous lymphomas by Kaudewitz and Braun-Falco the most valuable in this respect.

The book is reasonable value for money.

RONA M.MACKIE

Advances in Dermatology, Volume 7. EDITED BY J.P.CALLEN, M.V.DAHL, L.E.GOLITZ, H.T.GREENWAY JR AND L.A. SCHACHNER (1992). Chicago: Mosby. Pp. 352. ISBN 0-8151-1390-0. Price $59.95.

This American competitor of the Recent Advances series is useful to review and compare with Volume 9 of that series. This is an annual publication with specific areas delegated to one of the editors. Clinical research, dermatopathology, paediatric skin disease and dermatological surgery are the main areas of discussion in this volume, and at the end of each individual review there is an editor's comment. As I have previously suggested, these comments are perhaps rather too bland to be of any genuine value and are becoming more of a brief summary than a constructive or, indeed, a critical comment.

In the clinical research section there is an outstanding review by Sontheimer and colleagues of the clinical correlations and biological significance of antinuclear antibodies. There follow reviews on growth factors and cytokines, both of which are already slightly dated, such is the pace of advance in this area. The most interesting chapter in the section on dermatopathology is one on acoustic microscopy, with startlingly good photography to illustrate the value of this approach. The section on paediatric skin disease includes contributions on lichen sclerosus et atrophicus, prenatal diagnosis, and alopecia areata, the most timely and useful being that on prenatal diagnosis. Dermatological therapeutics is covered by chapters on extemporaneous compounding and phototherapy, and dermatological surgery by sections on chemical peeling, office sedation and superficial anatomy. An anaesthetist of my acquaintance, when shown the section on office sedation, expressed considerable concern about the translation of some of the recommended methods to the U.K. setting.

Overall, the book is pleasantly presented and is probably better value for money than that in the Recent Advances series.

RONA M.MACKIE

Books Received

The Dictionary of Modern Medicine. EDITED BY J.C.SEGEN (1992). Parthenon Publishing U.K. ISBN 1-85070-321-3. Price £48.00.

This is a reasonable-sized dictionary of medical terms which might be of use to a secretary or other member of staff without medical knowledge. The fact, however, that the spelling is American style may well deter some purchasers.

British Journal of Dermatology (1992) 127, 451.

News and Notices

10th Course for Consultants and Senior Registrars, St John's Institute of Dermatology, 11–14 February 1993, Brighton

Because this will be the tenth anniversary of the course for Consultants and Senior Registrars, with the support of Sandoz (U.K.), the programme will be entitled: 'Dermatological Management: the next ten years' and will, as usual, feature presentations from internationally recognized experts in relevant fields. There will also be the usual case presentation sessions and a social programme. The venue will be the Metropole Hotel, Brighton, Sussex. For further details please contact: Miss Teresa Brewer, Course Administrator, St John's Institute of Dermatology, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH. Telephone 071-922-8075. Fax 071-928-1428.

Joint Annual Meeting of the British Association of Dermatologists and the Canadian Dermatology Association, 6–10 July 1993, Oxford

The 73rd Annual Meeting of the British Association of Dermatologists will be a joint meeting with the Canadian Dermatology Association under the joint presidency of Dr William Gerstein (Canada) and Dr Terence Ryan (U.K.).

The meeting will be held in the Academic Centre at the John Radcliffe Hospital, Oxford from Tuesday, 6 July to Saturday, 10 July 1993.

Abstracts of papers and posters are invited for consideration by a Joint Abstract Selection Committee and those accepted as papers will be allotted 10 minutes for presentation plus time for discussion. Abstracts must be submitted on the official forms, which are available from: Miss Michelle Hardwicke, BAD Conference Office, 3 St Andrew's Place, London NW1 4LB, U.K.

The closing date for receipt of abstracts is Friday, 15 January 1993 but applicants should note that the Scientific Committee will be administered by the Canadian Dermatology Association and extra time, therefore, should be allowed for postage to Canada.

Instead of the usual clinical/CPC session held on Saturday morning, there will be a 'Celebration of Dermatology' to be held in the Churchill Hospital, Oxford. Further details will be published in the autumn edition of the BAD Newsletter.

ISBS Regional Workshop, 24–25 April 1993, Lübeck, Germany

A Regional Workshop of the International Society for Bioengineering and the Skin (ISBS), organized by the Departments of Dermatology, Universities of Lübeck, Germany, and Zürich, Switzerland, on 'Costmetic Dermatology—Facts and Fantasy', will be held on 24–25 April 1993 in Lübeck, Germany. For further information, please contact: Dr K.P.Wilhelm, Department of Dermatology, Medical University, Ratzeburger Allee 160, D 2400 Lübeck 1, Germany. Telephone (010) 49-451-5002515. Fax (010) 49-451-5002981.

Introductory Course on the Biology of the Skin, Cambridge, December 1992

The annual introductory course 'The Biology of the Skin' will be held in Fitzwilliam College, Cambridge, on 7–11 December 1992. This course is primarily for registrars and postgraduate students at an early stage of their training in dermatology but is open to other interested practitioners. Closing date for applications is 20 November, 1992. For further information please contact: the Conference Secretary (BoS), Postgraduate Medical Centre, Clinical School, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2SP, U.K. Telephone Cambridge (0223)-217105.

British Journal of Dermatology (1992) 127, 453–457.

Increased urine neopterin levels in psoriasis

C.C.HARLAND, R.P.WHITAKER,* J.L.BARRON* AND C.A.HOLDEN

*Departments of Dermatology and *Chemical Pathology, St Helier Hospital, Carshalton, U.K.*

Accepted for publication 20 June 1992

Summary

The production of neopterin closely reflects activation of T-lymphocyte-mediated immunity. Oxidized and reduced forms of urine neopterin were measured by reversed-phase ion-pair high-performance liquid chromatography in patients with moderate to severe chronic plaque psoriasis ($n = 40$), and in a heterogeneous group of patients ($n = 14$) with cutaneous T-cell malignancies (CTCM). Results were compared with healthy non-psoriatic control subjects ($n = 30$). Neopterin levels were repeated after a course of ultraviolet B therapy (UVB) plus topical tar or dithranol, or photochemotherapy (PUVA), in 12 psoriatic patients. Fully oxidized urine neopterin levels and neopterin/creatinine ratios were significantly elevated in the psoriatic group compared with controls ($P < 0{\cdot}002$, $P < 0{\cdot}05$) but not in the CTCM group. Both neopterin and its creatinine ratio were significantly reduced by treatment ($P < 0{\cdot}05$, $P < 0{\cdot}01$). Psoriasis area and severity index scores (PASI) correlated strongly with urine neopterin levels ($P < 0{\cdot}001$). These findings indicate that urine neopterin concentrations may be a marker of psoriatic disease activity, and further support the importance of activated T lymphocytes in the pathogenesis of psoriasis.

Pterins are a complex group of biological compounds.[1] Folates and pterins, members of the chemical group known as pteridines, share a characteristic ring structure, which can exist in different states of oxidation. Fully oxidized pterin species are fluorescent, and are responsible for the yellow pigment of butterfly wings (xanthopterin).

Neopterin and biopterin species are of immunological interest. Neopterin, whose biological role is uncertain, is released *in vitro* from macrophages and monocytes following interferon-γ stimulation, the latter being a product of activated T lymphocytes.[2] Thus, neopterin is a non-specific marker of T-cell activation, whilst the reduced form of biopterin (tetrahydrobiopterin) is said to be a marker of T-cell proliferation.[3,4] Neopterin measurements in biological fluids have been used for monitoring disease activity where there is activation of cellular immunity. Urine and serum neopterin levels have been found to be elevated in patients with malignancy,[5] viral infections,[6] autoimmune conditions,[7] renal allograft rejection,[8] and acquired immunodeficiency syndrome (AIDS).[9] Rising neopterin levels have been found to precede the development of graft-versus-host disease following bone marrow transplantation.[10]

Pterins have been overlooked in dermatological research, despite the fact that cellular immunity is important in the pathogenesis of many skin diseases. Two studies have indicated that pretreatment serum neopterin concentrations are not elevated in psoriatic patients,[11,12] but that they may reflect improvement or deterioration of psoriasis.[12] However, a small number of patients have been investigated, and the assays employed may have been insensitive because of a failure to discriminate between the oxidized and reduced forms of neopterin.

Although there is a close correlation between serum and urine pterin levels, urine neopterin concentration is one thousand times greater than serum concentration, thus facilitating its detection. In this study, urine neopterin concentration was measured before and after phototherapy in patients with chronic plaque psoriasis, an inflammatory skin disease in which T-cell activation is thought to play a role in pathogenesis.[13] As urine neopterin levels have been shown to be increased in lymphoid neoplasms, a heterogeneous group of cutaneous T-cell malignancies was also examined.[14] A specific high-performance liquid chromatography (HPLC) system for neopterin was employed.

Methods

Control subjects

A group of 30 healthy volunteers provided urine samples in order to establish a reference range for pterin

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Correspondence: Dr C.C.Harland, Department of Dermatology, St Helier Hospital, Wrythe Lane, Carshalton SM5 1AA, U.K.

concentrations. None suffered from psoriasis. Subjects were age- and sex-matched with the psoriatic patients.

Patients

Psoriasis. Forty patients with moderate to severe chronic plaque psoriasis were selected from the dermatology clinic for pretreatment urine pterin measurements. None of the patients had received ultraviolet therapy or systemic drug therapy for at least 4 weeks prior to treatment with topical dithranol, tar plus UVB, or PUVA. Pterin measurements were repeated at completion of a course of treatment in a subgroup of 12 patients (post-treatment). Clinical improvement was assessed by PASI.[15]

Cutaneous T-cell malignancy. A heterogenous group of 14 patients with cutaneous T-cell malignancy (CTCM) comprised individuals with cutaneous T-cell lymphoma (CTCL) or T-cell leukaemia with cutaneous deposits. Patients with CTCL, predominantly mycosis fungoides and Sézary syndrome, were selected from a specialist referral centre. Diagnoses had been confirmed by skin or lymph node biopsy, histology, immunocytochemistry and T-cell rearrangement studies of peripheral blood, skin or lymph nodes. Diseases were staged clinically (Stage I, cutaneous patches; II, plaques; III, tumour; IV, erythroderma; a, no lymph node involvement; b, nodal disease). Disease categories included mycosis fungoides ($n=8$), Sézary syndrome ($n=4$), lymphomatoid papulosis ($n=1$) and acute lymphoblastic leukaemia with cutaneous deposits ($n=1$), and covered a spectrum of clinical stages (Ia–IVb).

Exclusion criteria

Full blood count, renal and liver function were assessed for every subject. Patients were excluded from the study if there was evidence of any factor which might have influenced urine pterin concentrations: recent viral illness, other major illness, renal or hepatic dysfunction, medication with methotrexate, folate or multi-vitamins, or drugs which interfered with the HPLC assay (e.g. etretinate).

Pterin measurements

Twenty-millilitre aliquots of freshly voided urine were collected into light-protected containers with 1 mg/ml of sodium metabisulphite and disodium EDTA as antioxidant. Samples were frozen immediately at −70°C. After thawing, samples were centrifuged and injected directly on to a reversed-phase ion-pair HPLC system. The six pterin species, neopterin (N), biopterin (B), dihydroneopterin (NH_2), dihydrobiopterin (BH_2), tetrahydroneopterin (NH_4) and tetrahydrobiopterin (BH_4), were well separated on the HPLC system. N and B were detected by fluorescence. NH_2 and BH_2 were oxidized to N and B, respectively, using a post-column electrochemical conditioning cell and then detected by fluorescence. The reduced pterins BH_4 and NH_4 were detected by a dual coulometric electrode. The unknown pterin concentration (μg/l) was calculated by comparison with the standard peak heights. In an attempt to overcome

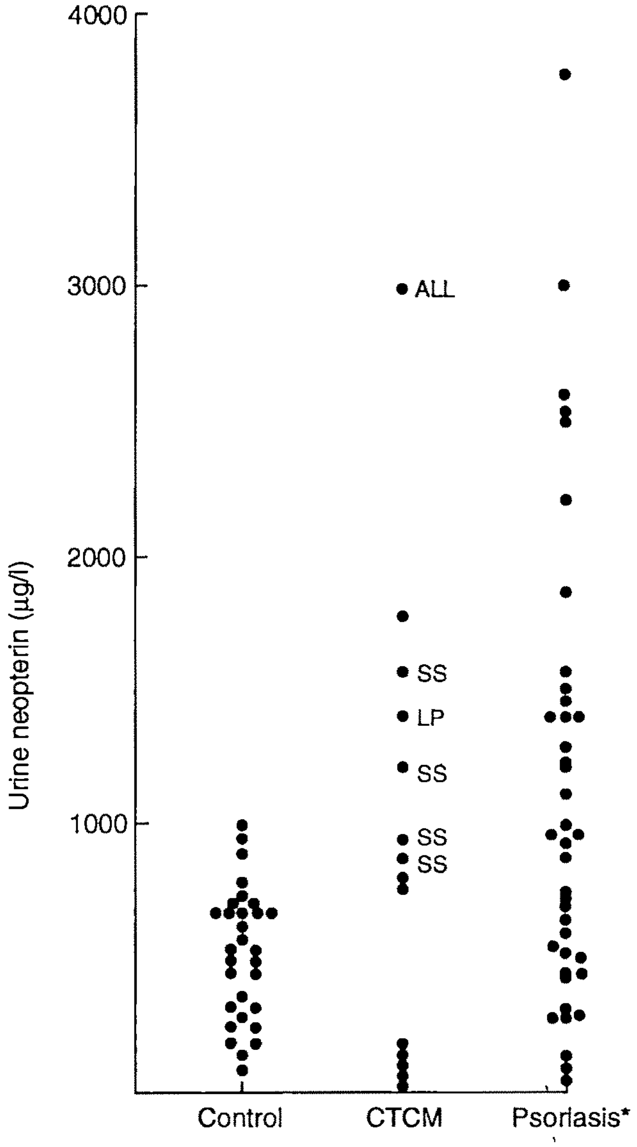

Figure 1. Urine neopterin levels for control subjects ($n=30$), cutaneous T-cell malignancies (CTCM) ($n=14$), and pretreatment psoriatic patients ($n=40$). ALL, acute lymphoblastic leukaemia; SS, Sézary syndrome; LP, lymphomatoid papulosis. *$P<0{\cdot}002$ compared with control subjects.

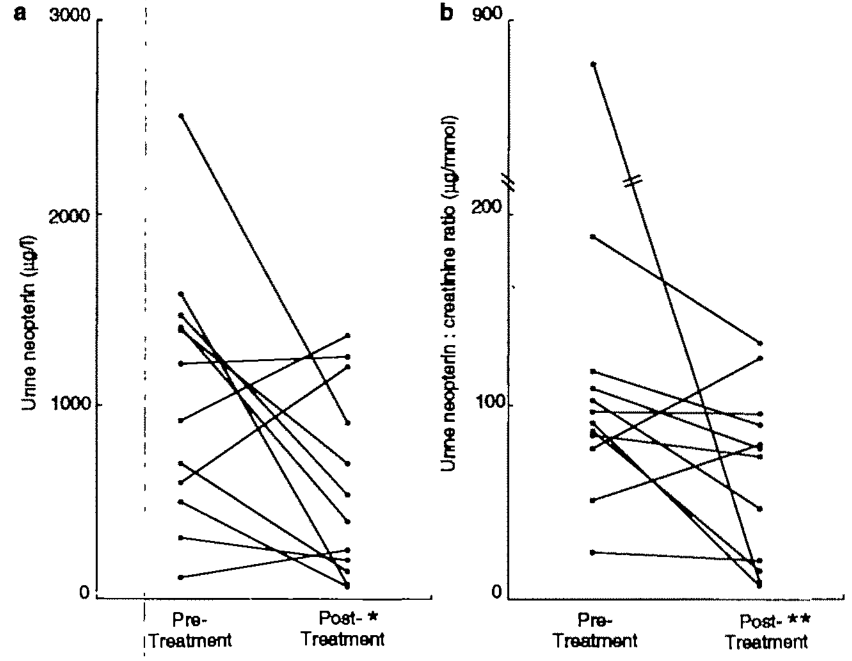

Figure 2. (a) Urine neopterin levels and (b) urine neopterin: creatinine ratio for psoriatic patients ($n=12$) pre- and post-treatment. $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

diurnal variability of urine concentration, urine pterins were expressed as a ratio with urine creatinine concentration (μg pterin/mmol creatinine). As it has been shown that excretion of pterins remains relatively constant throughout the day, the timing of urine collection was not standardized.[16]

Statistical analysis

Analysis for parametric data was used for pterin and creatinine concentrations within the control population (mean, standard deviation (SD); unpaired Student *t*-test for comparison between sexes, and for comparison of creatinine concentrations between groups). Non-parametric statistics were used for all other data (Wilcoxon's signed rank and Mann–Whitney *U*-tests for comparison of neopterin levels between groups; linear regression and Spearman's rank correlation coefficient for PASI scores versus urine neopterin and creatinine concentrations, and versus neopterin/creatinine ratios).

Results

Control group

The control group consisted of 30 subjects (17 male, 13 female; age range 20–60, mean 39·7 years) for which the urine neopterin range was 80–960 μg/l (mean 520, SD 220), and neopterin/creatinine ratio was 6–102 μg/mmol (mean 54, SD 24). There was no significant difference between the results in males and females. The range for urine creatinine concentration was 2·5–14·7 μmol/l (mean 8·35, SD 3·33).

Psoriasis

Pretreatment. Forty patients were included in the study (22 male, 18 female; age range 15–75, mean 37·7 years; PASI scores 2·0–28·8, mean 10·3). Urine neopterin levels (63–3800, mean 1122 μg/l), shown in Figure 1, and neopterin/creatinine ratios (8–880, mean 119 μg/mmol) were significantly elevated compared with the control group ($P<0{\cdot}002$; $P<0{\cdot}05$). The dihydroneopterin levels and dihydroneopterin/creatinine ratios were not significantly different from the control group and CTCM. The urine creatinine concentrations were higher than those of the control group (1–43·2 μmol/l, mean 12·5, SD 8·53; $P<0{\cdot}02$).

Post-treatment. Measurements were made after treatment of 12 patients. A clinical improvement was experienced by all patients (pretreatment PASI 4·4–14·4, mean 9·6; post-treatment 0–4·8, mean 1·3; $P<0{\cdot}01$). Urine neopterin and neopterin/creatinine ratios were significantly reduced following treatment ($P<0{\cdot}05$, $P<0{\cdot}01$) (Fig. 2).

PASI score correlation. There was a good correlation between pretreatment neopterin levels and PASI scores ($r=0{\cdot}53$, $P<0{\cdot}001$) (Fig. 3), and for pretreatment neopterin/creatinine ratios and PASI scores ($r=0{\cdot}37$, $P<0{\cdot}05$). A weak correlation existed for urine creati-

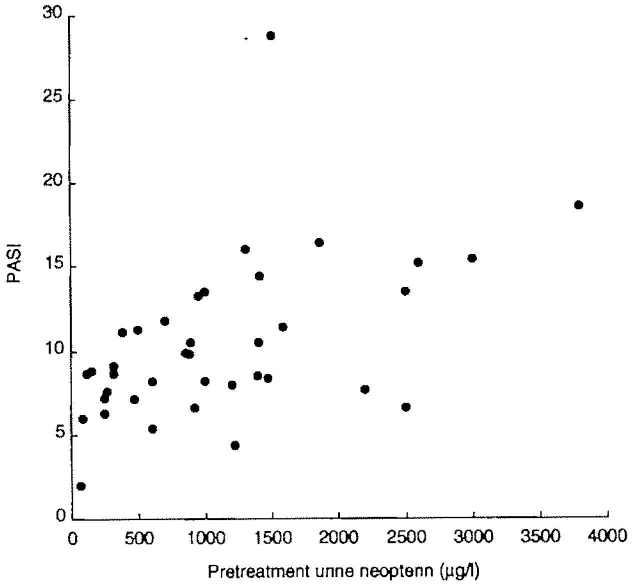

Figure 3. Comparison between urine neopterin levels and PASI scores for all pretreatment psoriatic patients ($n=40$). Spearman's rank correlation coefficient $r=0{\cdot}53$ ($P<0{\cdot}001$).

nine concentration and PASI scores ($r=0{\cdot}28$, $P=0{\cdot}077$).

CTCM

Fourteen patients were selected (five male; nine female; age range 32–82, mean 60 years). Pterin concentrations did not differ significantly from control subjects or psoriatic patients (Fig. 1), and bore no consistent relationship to the clinical stage of disease. However, the patient with acute lymphoblastic leukaemia (ALL) had a markedly elevated urine neopterin level. Patients with lymphomatoid papulosis (LP) and Sézary syndrome (SS) also had elevated neopterin levels (Fig. 1).

Other urine pterin results

There were no significant differences between control and patient groups, before and after treatment of psoriasis, for urinary dihydroneopterin (NH_2) concentrations. Other urinary pterins tested (NH_4, B, BH_2, BH_4) were generally undetectable.

Discussion

Urine neopterin levels and neopterin/creatinine ratios in non-psoriatic control subjects were similar to those found by other workers.[17] Both values were significantly elevated in patients with plaque psoriasis compared with non-psoriatic control subjects, and were reduced by treatment. Furthermore, PASI scores correlated strongly with neopterin levels. Urine neopterin and its creatinine ratio were not significantly elevated in patients with cutaneous T-cell malignancy. In particular, there was no correlation with the clinical stage of mycosis fungoides. However, neopterin levels were elevated in a subgroup of patients with Sézary syndrome, lymphomatoid papulosis and acute lymphoblastic leukaemia with skin infiltration.

Neopterin correlated better with PASI scores than with urine neopterin/creatinine. This observation may be the result of higher and more variable urine creatinine concentrations found in psoriatic patients. It is not clear why urine creatinine concentration should differ in psoriasis; there was a tendency for the concentration to be greater in patients with clinically more severe psoriasis. Dehydration due to excessive transepidermal fluid loss is unlikely to have affected the results significantly, and none of the patients was taking systemic therapy, such as cyclosporin, which could have interfered with creatinine excretion. Early morning or 24 h urine samples, or serum measurements, may have been preferable, despite the evidence that pterin concentrations do not vary greatly during the day.[16]

Other workers have shown variable results for neopterin measurements in psoriatic patients.[11,12] Neopterin levels have not previously been shown to be elevated, or to correlate with pre-treatment PASI scores. In contrast with previous work, this study indicates that neopterin estimation can be sensitive enough to demonstrate increased T-cell activation in chronic plaque psoriasis. There may be several explanations for this apparent sensitivity. Firstly, the fully oxidized form of neopterin was separated from other neopterin species. Other techniques tend to measure all reduced and oxidized species, although the fully oxidized molecule is probably the best marker of T-cell activation.[2] Secondly, a larger number of patients was selected. Thirdly, urine neopterin concentration, as opposed to serum concentration, was determined, which may reflect an integrated release of neopterin. Urine pterin concentrations are also easier to analyse. Both urine neopterin and its creatinine ratio were evaluated. Some workers claim that the pterin compound alone is required for urine analysis,[18] whereas others use creatinine ratios in order to overcome variations in urine concentrations.[5] This current data suggests that both measurements are sensitive, although neopterin alone correlated better with PASI scores.

These findings are consistent with the current evidence for the role of cell-mediated immunity in the pathophysiology of psoriasis. Early psoriatic lesions have been reported to show an infiltration of activated helper T cells.[19,20] Activated T cells release interferon-γ, which has been identified in the serum and suction-blister fluid of psoriatic lesions.[21,22] Furthermore, interferon-γ production in psoriatic epidermis has been confirmed using polymerase chain-reaction techniques.[23] Interferon-γ is pro-inflammatory in skin and may also be important for the trafficking of T lymphocytes into lesional psoriatic epidermis.[13] Its *in vivo* effects on psoriatic skin are variable, but it has been shown to induce psoriasis at the site of injection.[24] As large amounts of neopterin are produced by human monocytes/macrophages on stimulation with interferon-γ, and increased neopterin values indicate endogenous production of interferon-γ,[2] serum and urine neopterin concentrations might be expected to be elevated in psoriatic patients and to fall with treatment.

The practical value of neopterin for monitoring skin diseases has yet to be established. A study of serial serum or urine neopterin measurements is required to evaluate their clinical role for assessing disease activity or therapy for individual psoriatic patients. If T-cell activation plays an important part in the initiation of psoriatic plaques, neopterin may be a useful predictor of disease relapses. Further studies on other inflammatory or malignant skin diseases would be of interest. However, neopterin is unlikely to be of use in the assessment of mycosis fungoides.

Acknowledgment

We are grateful to Dr Neil Smith for allowing us to select patients from the Skin Tumour Unit, St Thomas's Hospital, London.

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British Journal of Dermatology (1992) 127, 458–462.

T lymphocytes bearing the $\gamma\delta$ T-cell receptor: a study in normal human skin and pathological skin conditions

M.ALAIBAC, J.MORRIS, R.YU AND A.C.CHU
Unit of Dermatology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K.

Accepted for publication 20 May 1992

Summary

The aims of this study were to investigate the presence of $\gamma\delta$ T cells in normal human skin, and the possible role of these cells in cutaneous reactions. Twenty-eight samples of normal skin from various sites, and 52 biopsies from inflammatory and neoplastic skin conditions were investigated by immunohistochemical techniques. In normal human skin $\gamma\delta$ T cells were infrequently seen in the epidermis and dermis. In the inflammatory and neoplastic dermatoses, $\gamma\delta$ T cells were occasionally present, accounting for 0–5% of CD3$^+$ cells in most of the biopsies examined. In one case of pityriasis lichenoides chronica and one case of lichen planus $\gamma\delta$ T cells were found to be increased, accounting for 15% of the CD3$^+$ cells in each case. Dermal $\gamma\delta$ T cells were markedly increased in three of six cases of Langerhans cell histiocytosis, with up to 30% of dermal CD3$^+$ cells showing positive staining to an anti-T-cell receptor $\gamma\delta$ monoclonal antibody. In two of these cases $\gamma\delta$ T cells were seen in both the dermis and the epidermis. In two further cases dermal $\gamma\delta$ T cells were not a prominent feature, but small clusters of epidermal $\gamma\delta$ T cells were observed.

T cells bearing the $\gamma\delta$ T-cell receptor are thus not a major feature of normal human epidermis, unlike the murine system, where the great majority of epidermal lymphocytes express the $\gamma\delta$ T-cell receptor. The finding of numerous $\gamma\delta$ T cells in Langerhans cell histiocytosis suggests a possible functional relationship between $\gamma\delta$ T cells and Langerhans cells or possibly a pathogenic role of these cells in Langerhans cell histiocytosis.

Two distinct types of CD3-associated T-cell receptors (TCR) have now been identified. The first type, expressed by the great majority of human T lymphocytes in peripheral lymphoid organs, blood, and lymphomas, is a heterodimer composed of α and β chains. $\alpha\beta$ T cells express either CD4 or CD8 molecules and recognize antigens in association with MHC class II or class I determinants, respectively.[1,2] The second type of receptor, found on the surface of a small percentage of thymocytes and peripheral T cells (1–5%), is a heterodimer composed of γ and δ chains.[3] In humans, TCR $\gamma\delta$ was initially identified on T-cell lines that shared two main phenotypic characteristics, namely, the lack of reactivity with monoclonal antibodies (MAb) directed against TCR $\alpha\beta$ and a 'double negative' (CD4$^-$CD8$^-$) phenotype.[4]

In the last few years some evidence has been obtained suggesting a possible function for these cells. TCR $\gamma\delta$ appears to represent the first CD3-associated TCR in T-cell ontogeny and may represent the ancestral TCR in evolution.[5] In adult mice, $\gamma\delta$ T cells are regionally enriched in the epidermis,[6] and in the intestinal epithelium,[7] where they represent a significant immigrant cell population. Recent experiments have shown that a significant proportion of $\gamma\delta$ T cells are able to recognize both mycobacterial antigens and autologous heat shock or stress proteins (HSPs), a family of conserved proteins induced or increased in all cells and organisms after exposure to various types of injury.[8,9] These observations, together with the ability of $\gamma\delta$ T cells to display different types of cytotoxicity,[10] has led to the hypothesis that $\gamma\delta$ T cells represent a primitive surveillance system that monitors cell integrity and destroys cells altered by transformation, or invading micro-organisms at epithelial surfaces.[11,12]

The situation regarding selective epidermal or intestinal tissue distribution of $\gamma\delta$ T cells in man is controversial, as to date such a compartmentalization has not been consistently observed.[13–15] The aims of this study were to investigate the regional importance of $\gamma\delta$ T cells

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Correspondence: Dr M.Alaibac.

Presented at the European Society for Dermatological Research 21st Meeting, Copenhagen, 1–4 June 1990.

Table 1. Normal human skin specimens investigated

Specimen site	Number of specimens
Breast	4
Eyelid	4
Face	4
Neck	4
Hair-margin	4
Trunk	4
Abdomen	4

in normal human skin, and the possible role of these cells in cutaneous reactions. We used an anti-γδ reactive MAb on frozen tissue sections to compare the frequency and the location of these cells in normal human skin and a number of inflammatory and neoplastic dermatoses, including six cases of Langerhans cell histiocytosis (LCH).

Methods

Specimen collection

Twenty-eight samples of normal skin from different body regions were obtained from plastic surgical operations or from cosmetic removal of benign tumours. The sites of the specimens of normal skin are listed in Table 1.

Twenty-eight biopsies of inflammatory dermatoses, including six of Langerhans cell histiocytosis (LCH), and 23 biopsies of neoplastic skin lesions, were examined. Lesions were diagnosed using standard clinical and histological criteria. Specimens were embedded in OCT compound (Lab Tek Division, Miles Laboratories, Naperville, IL, U.S.A.), snap frozen in liquid nitrogen, vertically sectioned at 4–6 μm thickness and placed on poly-L-lysine pretreated slides.

Monoclonal antibodies

Sections were stained as described below with the MAbs shown in Table 2. TCRδ1 MAb binds to a δ constant region determinant on the γδ heterodimer[16] and reacts with all known TCR γδ-bearing cells, clones and cell lines.

Immunohistochemical staining

Frozen sections of normal and pathological samples were air-dried at room temperature for 24 h and fixed in acetone for 2 min at 4°C. For staining, alkaline phosphatase anti-alkaline phosphatase complex (APAAP) and indirect immunoperoxidase methods were used as previously described.[17,18] Briefly, after washing in Tris-buffered saline (TBS) pH 7·6, sections were incubated with MAbs at appropriate dilution for 12 h at 4°C. After three washes in TBS, rabbit anti-mouse Ig (Dakopatts)(1:25 dilution) was applied for 30 min, and after washing in TBS the sections were incubated with APAAP, (Dakopatts) (1:20 dilution) for 30 min. Cytospin preparations of peripheral blood mononuclear cells served as positive controls. As negative controls, the first-step reagent was omitted or substituted with normal mouse serum. The alkaline phosphatase reaction was developed in 0·1 M Tris HCl buffer, pH 8·2, with naphthol-AS-MX-phosphate (Sigma Chemical Co., St Louis, MO, U.S.A.) and fast red TR Salt (Sigma) containing 1 mM Levamisole (Sigma) to block endogenous alkaline phosphatase activity. Positive cells stained a carmine-red colour. Sections were counterstained with Mayer's haematoxylin and the slides mounted in Apathy's medium.

Cell counts

In normal human skin, 5–10 random sections of each specimen were stained with either anti-CD3 MAb or

Table 2. Panel of monoclonal antibodies used in the study

Antibody	Directed against	Source	Dilution
TCRδ1	γδ TCR -δ chain	T cell Sciences, Cambridge MA, U.S.A.	1:20
βFR	αβ TCR-β chain	T cell Sciences, Cambridge MA	1:20
Dako T3	CD3	Dakopatts, Glostrup, Denmark	1:40
Dako T4	CD4	Dakopatts, Glostrup, Denmark	1:25
Dako T8	CD8	Dakopatts, Glostrup, Denmark	1:25
Dako T6	CD1a	Dakopatts, Glostrup, Denmark	1:25
Dako Macrophage	Macrophages	Dakopatts, Glostrup, Denmark	1:20

anti-$\alpha\beta$ TCR MAb, and 20–30 random sections were stained with anti-$\gamma\delta$ TCR MAb. These sections were examined by light microscopy at × 100 magnification, and the density of intraepidermal CD3$^+$, $\alpha\beta^+$ and $\gamma\delta^+$ cells was calculated as the number of positively stained cells per linear centimetre (cm) of epidermis. In the pathological specimens, serial frozen sections were examined by light microscopy at × 400 magnification. In these specimens the presence of $\gamma\delta$ T cells was calculated as a percentage of the total number of T cells (i.e. CD3$^+$ cells) in the dermal and epidermal components.

Results

Presence of $\gamma\delta$ T cells in normal human skin

Normal human skin from various body regions demonstrated CD3$^+$ cells in both the epidermis (5–6 cells per linear cm of epidermis) and dermis. T cells bearing the $\alpha\beta$ TCR represented the dominant subset in both the epidermal and dermal compartments. Occasional TCR $\gamma\delta$-bearing cells were found in both the epidermis (one cell per 20 linear cm of epidermis) and the dermis, where they were predominantly detected in perivascular areas. There was no regional variation of $\gamma\delta$ T-cell density in the different body sites examined.

Presence of $\gamma\delta$ T cells in inflammatory cutaneous lesions

In the inflammatory dermatoses investigated, the percentage of $\gamma\delta$-bearing T cells in the dermal and epidermal infiltrate was extremely low, accounting for less than 2% of CD3$^+$ cells in most of the biopsies examined (Table 3). The highest percentages of $\gamma\delta$ T cells were observed in one case of pityriasis lichenoides chronica and one case of lichen planus, where 15% of the CD3$^+$ cells stained with TCRδ1.

Table 3. Inflammatory skin conditions investigated—relative percentages of $\gamma\delta$ T cells

Dermatoses	Number of cases	% +ve
Viral wart	5	0–2
Lichen planus	3	0, 2, 15
Pityriasis lichenoides chronica	3	1, 1, 15
Graft-versus-host disease	2	0, 2
Lichen aureus	2	0, 2
Erythroderma	2	0, 1
Discoid lupus erythematosus	2	0, 1
Parapsoriasis-en-plaque (small plaque)	1	0
Granuloma annulare	1	2
Lymphocytoma cutis	1	1

Table 4. Neoplastic skin conditions investigated—relative percentages of $\gamma\delta$ T cells

Dermatoses	Number of cases	% +ve
Squamous cell carcinoma	10	0–2
Basal cell carcinoma	6	0–1
Cutaneous T-cell lymphoma	4	0–2
Bowen's disease	2	1, 2
Hodgkin's disease	1	5

Table 5. $\gamma\delta$ T cells in Langerhans cell histiocytosis.

Patient	% $\gamma\delta$ T cells in the dermis	Presence of clusters of $\gamma\delta$ T cells in the epidermis
1	30	+
2	1	–
3	5	+
4	25	+
5	5	+
6	30	–

Presence of $\gamma\delta$ T cells in neoplastic cutaneous lesions

The percentage of $\gamma\delta$ T cells in the dermal and epidermal infiltrate of the neoplastic cutaneous lesions investigated ranged between 0 and 5% of CD3$^+$ cells (Table 4).

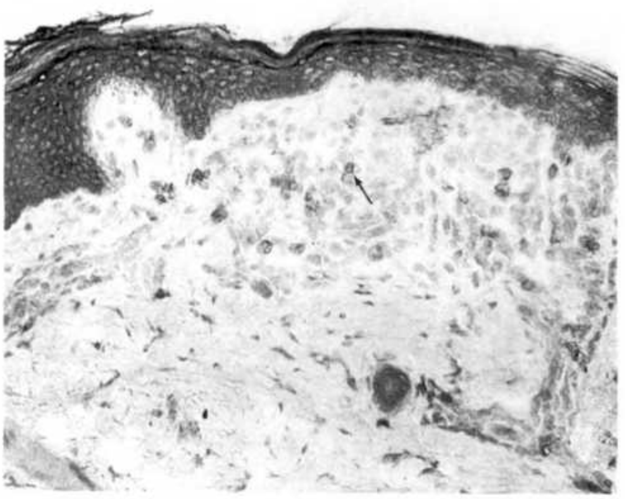

Figure 1. APAAP staining of lesional skin of LCH. Numerous $\gamma\delta$ T cells are present in the dermal infiltrate. Arrow indicates a representative positive $\gamma\delta$ T cell (× 100).

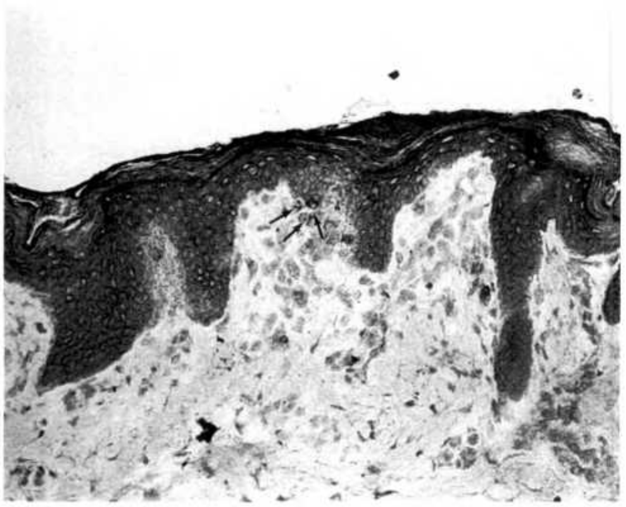

Figure 2. APAAP staining of lesional skin of LCH. A cluster of $\gamma\delta$ T cells (arrows) invading the epidermis is shown (× 200).

Presence of $\gamma\delta$ T cells in Langerhans cell histiocytosis

In six cases of LCH, $\gamma\delta$ T cells were observed in varying percentages (Table 5). In three cases, the percentage of $\gamma\delta$ T cells in the dermal infiltrate was up to 30% of the $CD3^+$ cells (Fig. 1). In two of these cases $\gamma\delta$ T cells showed evidence of epidermotropism (Fig. 2). In the remaining three cases the percentage of $\gamma\delta$ T cells varied from 1 to 5% of $CD3^+$ cells, and in two of these cases small clusters of epidermal $\gamma\delta$ T cells were observed.

Discussion

In this study we analysed normal human skin and some inflammatory and neoplastic skin lesions for the presence of $\gamma\delta$ T cells.

In normal human skin, $\gamma\delta$ T cells were infrequently seen in both the epidermal and dermal compartments, and no regional variation was observed. Our observations are in agreement with previous reports[13–15] and emphasize the difference between man and mouse, where the great majority of epidermal lymphocytes express TCR $\gamma\delta$.

In our study, we demonstrated that cells expressing TCR $\gamma\delta$ are not a major feature in most of the inflammatory and neoplastic dermatoses investigated, accounting for less than 5% of $CD3^+$ cells. The percentages of $CD3^+$ cells expressing $\gamma\delta$ TCR in these conditions were the same as would be expected in the blood. These results are consistent with previous reports[13,19] and suggest that generally there is no selective recruitment of $\gamma\delta$ T cells to either reactive or malignant skin lesions. It has been suggested that $\gamma\delta$ T lymphocytes could play an important role in the immune surveillance of epithelia against malignant cells,[4,12] but our findings do not substantiate this hypothesis in the neoplastic dermatoses we have investigated. However, the picture was different in LCH.

LCH embraces disorders previously called histiocytosis X, Letter–Siwe disease, Hand–Schüller–Christian disease and eosinophilic granuloma.[20] This term is applied to a clinically heterogeneous group of diseases characterized by the infiltration of one or more organs with cells that have the morphological, ultrastructural, histochemical and immunohistochemical characteristics of epidermal Langerhans cells.[21] In this study we observed an increased percentage of $\gamma\delta$ T cells in both the dermal and epidermal infiltrate in some cases of LCH. The reasons for this infiltration by $\gamma\delta$ T cells are open to speculation. The prominence of $\gamma\delta$ T cells in LCH may indicate a functional interaction between LCH cells and $\gamma\delta$ T cells. The $\gamma\delta$ T cell may be a bystander cell, or may be accumulating as a result of an inductive influence of pathological Langerhans cells, possibly through cytokine production, or may represent a host immune response to LCH. To date, few links have been demonstrated between Langerhans cells and $\gamma\delta$ T cells. It has been observed that $\gamma\delta$ T cells are able to recognize antigens in association with MCH class-I like molecules.[22,23] CD1a and CD1c molecules, which are antigens expressed on the surface of Langerhans cells both in normal human skin and in LCH,[24] belong to the MHC class I-like proteins in man, and it has been suggested that these molecules could interact with $\gamma\delta$ T cells.[25] Such interactions between Langerhans cells and $\gamma\delta$ T cells have been observed in mice, where a recent investigation has shown that Langerhans cells stimulate lymphokine secretion and proliferation of murine epidermal TCR $\gamma\delta^+$ cells.[26] An increase of $\gamma\delta$ T cells in association with an overexpression of CD1 antigens has recently been described in colonic epithelium,[27] and in the epidermis of lepromin skin tests.[28] Thus, overexpression of CD1 might account for the higher percentage of $\gamma\delta$ T cells in LCH. Evidence against this hypothesis is the low number of $\gamma\delta$ T cells observed in certain conditions characterized by an increase of CD1 expressing cells, such as lichen planus[13] or bronchogenic carcinomas.[29]

Additional functional investigations are required in order to determine the relationships, if any, between $\gamma\delta$ T lymphocytes and both normal and pathological Langerhans cells. The varying number of $\gamma\delta$ T cells in the cases of LCH examined suggests that further studies should be performed to assess whether their presence or absence bears any meaningful relationship to disease activity and prognosis.

Acknowledgments

The work was supported by a grant from the Marcus Nunn Histiocytosis X Research Fund. Mauro Alaibac is a M.U.R.S.T. Research Fellow. Raymond Yu is the Marcus Nunn Research Fellow.

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British Journal of Dermatology (1992) 127, 463–469.

Aberrant expression of p53 tumour-suppressor protein in non-melanoma skin cancer

J.M.McGREGOR, C.C-W.YU,* E.A.DUBLIN,* D.A.LEVISON* AND D.M.MACDONALD*

*Departments of Dermatology and *Histopathology, Guy's Hospital, London SE1 9RT, U.K.*

Accepted for publication 9 June 1992

Summary Expression of the cellular p53 tumour-suppressor protein was examined in 78 epidermal tumours, including basal and squamous cell carcinomas, keratoacanthomas, solar keratoses, Bowen's disease and viral warts. An immunohistochemical study was employed using the antibody CM-1, raised against recombinant human p53 protein. Positive staining for p53, not detectable in normal cells because wild-type p53 is rapidly degraded, reflects abnormal stabilization of p53 protein, and in many cases suggests p53 gene mutation.

p53 immunoreactivity was not observed in normal skin or in viral warts. In contrast, positive staining for CM-1 was seen throughout the tumour in the majority of basal and squamous cell carcinomas and in Bowen's disease. Immunoreactivity to p53 was also observed in the majority of keratoacanthomas and solar keratoses, but was confined to areas of dysplastic basal epithelium. This study demonstrates that accumulation of p53 protein, suggestive in many cases of p53 gene mutation and hence loss of tumour-suppressor function, may occur as an important early step in the development of diverse epidermal cancers.

p53 gene mutations, which are located on the short arm of chromosome 17, band 13,[1] are the most frequent genetic alterations found in human malignancies.[2,3] Molecular studies of diverse cancers reveal frequent loss of both p53 alleles, one through deletion and the other through point mutation,[2,4] consistent with the theory that this locus encodes a tumour-suppressor protein[5] and that loss of function at this locus is an important step in tumorigenesis.[6]

Accumulating evidence suggests a critical role for wild-type p53 in the negative control of cell cycling and tumour progression.[6] Plasmids encoding wild-type p53 inhibit the transformation of rodent cells by known oncogenes[7,8] and have been shown to abolish the malignant phenotype of some human tumour cells.[9,10] Although the precise mechanism by which p53 protein modulates cell proliferation is not known, its nuclear localization suggests that it may be directly involved in the regulation of gene expression. p53 is a DNA-binding protein[11] and inactivating mutations of the p53 gene compromise the ability of the p53 protein to bind DNA.[12] Furthermore, wild-type p53 has been shown to down-modulate the activity of several promoter genes, including *c-fos*, which are normally involved in stimulation of cell proliferation.[13]

It has recently been suggested that p53 protein may also participate in the inhibition of replicative DNA synthesis which follows DNA damage,[14] including UV-induced damage.[15] Because replicative arrest may allow optimal repair of damaged DNA, and thus prevent the propagation of potentially harmful mutations, it is suggested that failure to enter this arrest phase, through loss of p53 function, may play an important part in the development of cancer.[14]

Inactivating p53 gene mutations, although predominantly confined to four regions highly conserved amongst vertebrate species,[16] may occur at numerous sites through point mutation, deletion or insertion.[2] Molecular analysis of p53 mutations in cancers of similar aetiology, however, suggests that specific carcinogens may leave their fingerprints on the human genome.[17] For example, G to T mutations at codon 249 of the p53 gene are found almost exclusively in hepatocellular carcinomas from areas where aflatoxin is endemic,[18] and CC to TT double base changes, virtually pathognomonic of UV-induced damage, have been found uniquely in epidermal squamous cell carcinomas.[19] These data provide compelling evidence for a direct role for UV-light in mutation of the p53 gene, and

Correspondence: Dr J.M.McGregor, Department of Dermatology, Guy's Hospital, London SE1 9RT, U.K.

support epidemiological studies which suggest that UV-light is an important aetiological factor in squamous cell tumorigenesis.[20]

Non-melanoma skin cancer provides an ideal model in which to study the role of p53 mutation in tumorigenesis. Benign, premalignant and malignant epidermal tumours on sun-exposed sites are readily available for examination. We have employed an immunohistochemical technique using CM-1, a recently characterized polyclonal antiserum to recombinant human p53,[21] to examine p53 immunoreactivity in human epidermal cancers *in vivo*. Cellular p53 protein, which is rapidly degraded,[22] is undetectable by immunohistochemistry in normal cells.[23] In human tumour cells in diverse tissues, however, elevated levels of p53 protein are consistently found[3,23,24] reflecting abnormal stabilization of p53 protein in these cells, in many cases through p53 gene mutation;[25] p53 immunoreactivity thus provides some evidence for mutation in the p53 gene. Seventy-eight epidermal tumours were examined, including solar keratoses, Bowen's disease, viral warts, keratocanthomas, basal cell and squamous cell carcinomas.

Methods

Material, tissue processing and immunohistochemical procedures

Seventy-eight epidermal tumours were studied, comprising 22 squamous cell carcinomas, 24 basal cell carcinomas, nine solar keratoses, five cases of Bowen's disease, 12 keratoacanthomas and six viral warts. In addition, skin biopsies of normal abdominal skin were obtained from six healthy adult volunteers. For each patient, following a detailed history and physical examination, the likely contribution of UV-light to the aetiology of the tumour was estimated.

Specimens were excised under local anaesthesia, formalin-fixed and subsequently embedded in paraffin wax prior to sectioning. The rabbit polyclonal antibody CM-1, raised against recombinant human p53[21] was employed for immunostaining as previously described.[23] Briefly, 4-μm sections from each specimen were mounted on poly-L-lysine-coated glass slides (Sigma diagnostics), air-dried overnight (27°C), then de-waxed and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in methanol. Non-specific binding was blocked by subsequent incubation with 5% normal porcine serum diluted in phosphate-buffered saline (PBS). A streptavidin–biotin–peroxidase technique was employed using CM-1 at a dilution of 1:1500 as the primary antibody, and a biotinylated porcine anti-rabbit serum (Dakopatts, High Wycombe, U.K.) as the secondary antibody. Peroxidase activity was visualized as a grey/black reaction product using a chromogen of 25 mg 3′-3′ diaminobenzidine, 1·25 ml 1% cobalt chloride, 1 ml 1% nickel ammonium sulphate and 100 μl of 30 volume hydrogen peroxide in 100 ml PBS. Sections were counterstained with nuclear fast red, dehydrated, cleared and mounted.

Tissue from a well characterized breast carcinoma known to express aberrant p53 was included as a positive control in each assay.[21] Appropriate negative controls consisted of substitution of the primary polyclonal antibody with PBS. Our group has previously verified the specificity of this technique for p53 immunoreactivity by incubation of sections with the immunizing p53 peptide prior to staining: this abolished staining in positive control sections.[26]

Results

Results of immunostaining are presented in Table 1. Immunoreactivity to p53 was visualized as grey/back nuclear staining as seen in the positive control case of breast carcinoma. Nuclear staining intensity was classified as either negative (pink), weak (grey) or strong (black); we observed very little cytoplasmic staining with CM-1 using this protocol. Control sections incubated with PBS gave consistently negative results.

p53 immunoreactivity in malignant and premalignant epidermal tumours (Table 1)

p53 immunoreactivity was observed in 50% (11 of 22) of squamous cell carcinomas and 46% (14 of 24) of basal cell carcinomas. In the majority of these tumours, strong positive nuclear staining was seen throughout the thickness of the tumour (Figs 1 and 2). Aberrant expression of p53 protein was also seen in 80% (4/5) of Bowen's disease and in 55% (5/12) of solar keratoses. In solar keratoses positive staining was confined to areas of dysplastic basal epithelium (Fig. 3).

p53 immunoreactivity in benign epidermal tumours and normal skin (Table 1)

p53 immunoreactivity was not seen in normal skin or in viral warts. In contrast, p53 immunoreactivity was

Table 1. p53 immunoreactivity in epidermal skin cancer

Tumour	Number	Negative (%)	Weakly + ve (%)	Strongly + ve (%)	Total + ve (%)
Solar keratosis	9	4 (45)	2 (22)	3 (33)	5 (55)
Bowen's disease	5	1 (20)	3 (60)	1 (20)	4 (80)
Keratoacanthoma	12	4 (36)	5 (40)	3 (25)	8 (64)
Squamous cell carcinoma	22	11 (50)	4 (18)	7 (32)	11 (50)
Basal cell carcinoma	24	10 (46)	4 (16)	10 (40)	14 (46)
Viral wart	6	6 (100)	0	0	0
Normal skin	6	6 (100)	0	0	0

observed in 64% (8/12) of keratoacanthomas. The staining pattern seen in these tumours was consistently different from that in squamous cell carcinoma: p53 immunoreactivity in keratoacanthomas was observed predominantly in basal keratinocytes (Fig. 4).

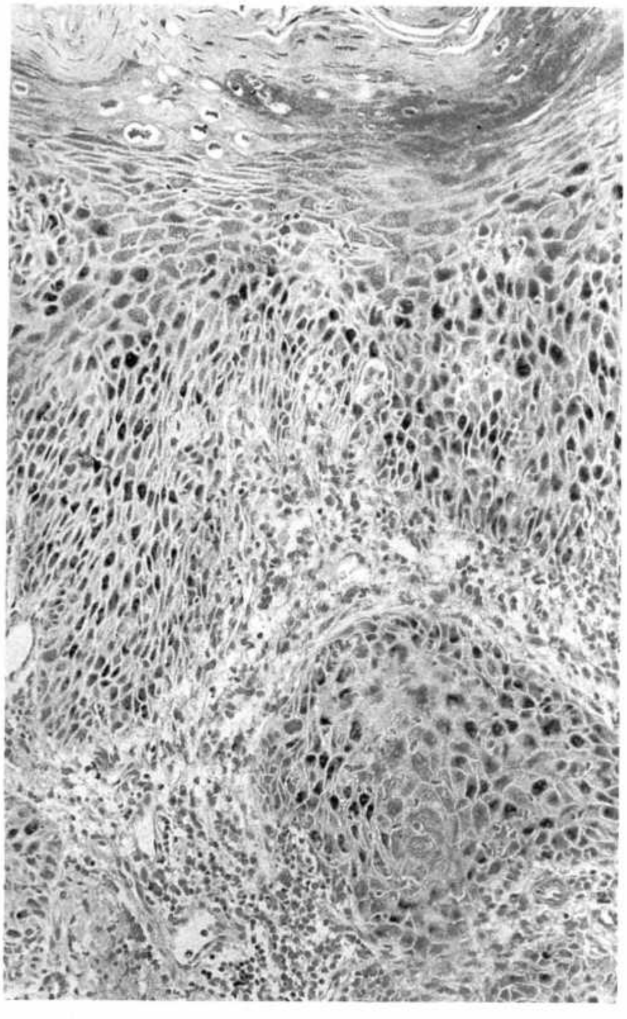

Figure 1. Paraffin section of squamous cell carcinoma stained for CM-1 (counterstained with nuclear fast red). Strong nuclear p53 immunoreactivity is seen throughout the thickness of the tumour. Only the superficial keratinizing layers show no p53 staining (× 64).

p53 immunoreactivity and sun-exposure (Table 2)

In 4/22 squamous cell carcinomas UV-light did not appear to be a significant aetiological factor: in two cases tumours arose on non-sun-exposed skin, in one case the

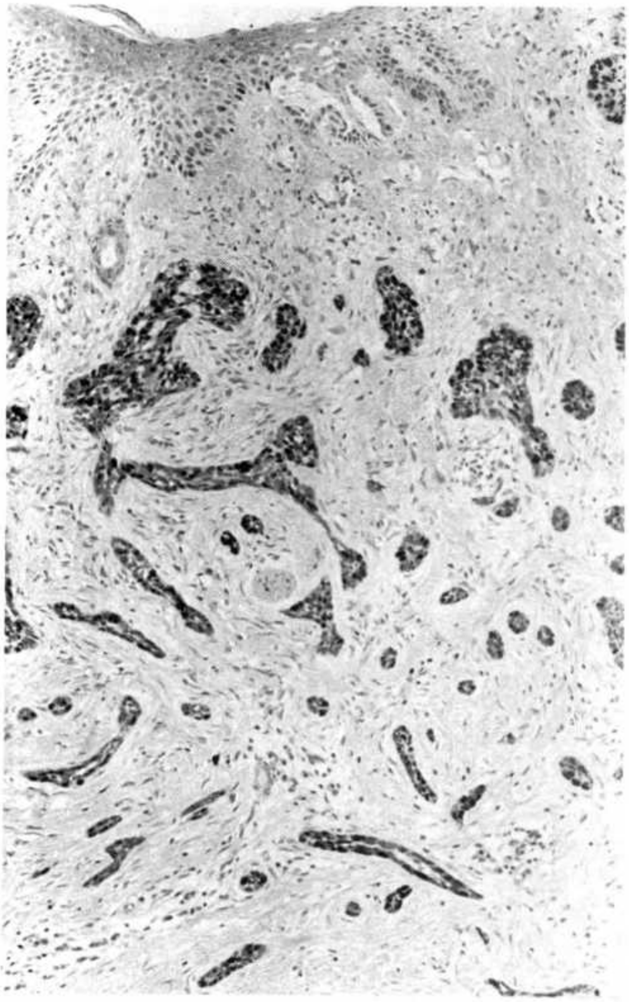

Figure 2. Paraffin section of basal cell carcinoma stained for CM-1 (counterstained with nuclear fast red). Strong p53 immunoreactivity is seen throughout the tumour. p53 immunoreactivity is also seen in dysplastic basal keratinocytes in overlying sun-damaged epidermis (× 32).

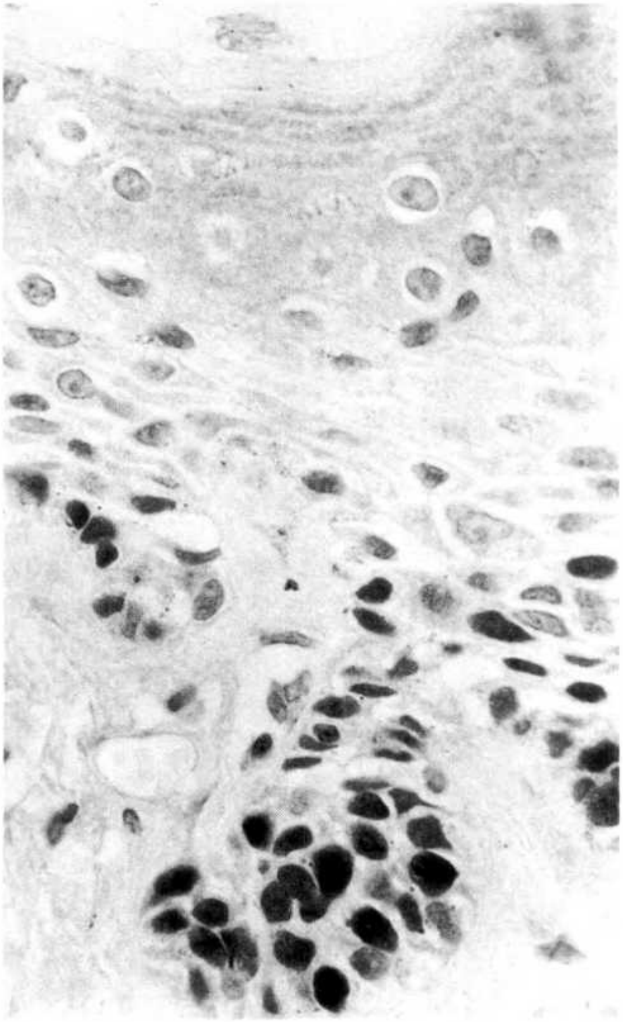

Figure 3. Paraffin section of solar keratosis stained for CM-1 (counterstained with nuclear fast red). Strong p53 immunoreactivity is seen predominantly in dysplastic basal keratinocytes of solar keratoses, compared with full thickness staining seen in Bowen's disease (not shown) (× 100).

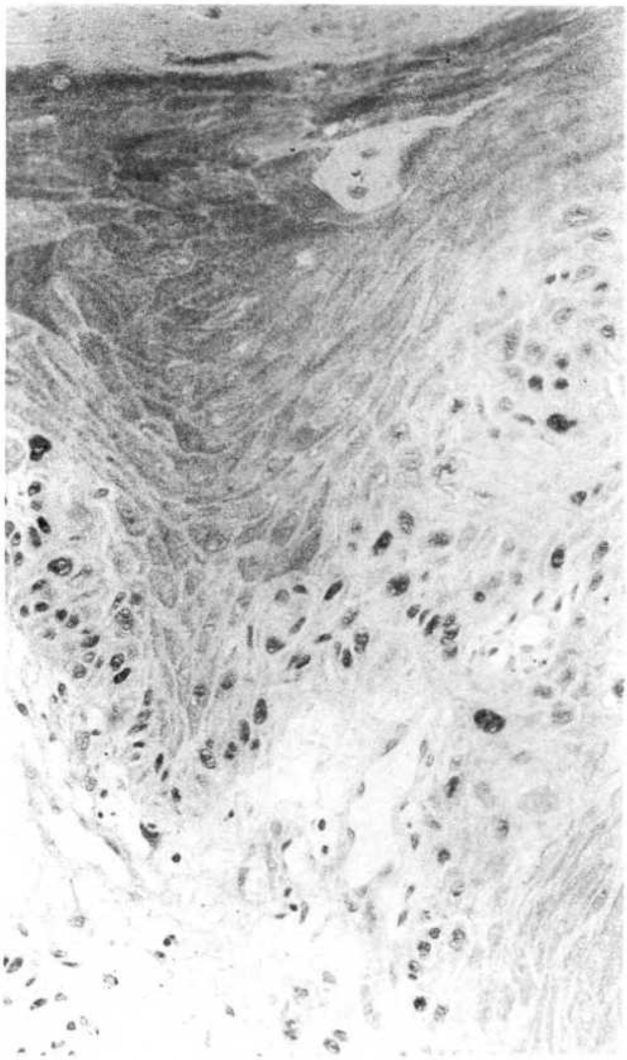

Figure 4. Paraffin section of keratoacanthoma stained with CM-1 (counterstained with nuclear fast red). p53 immunoreactivity is confined to basal keratinocytes in these tumours in contrast with full thickness staining observed in squamous cell carcinomas (Fig. 1). (× 80).

tumour arose in a long-standing venous ulcer and in another the tumour was excised from the thigh of a patient with keratitis, ichthyosis and deafness syndrome (a condition associated with squamous cell tumours[27]). p53 immunoreactivity was observed in a similar proportion of squamous cell carcinomas with and without a history of UV exposure, although in only one non-UV-induced tumour was strong immunoreactivity observed.

Although aetiological factors other than UV-light were identified in seven cases of basal cell carcinomas

Table 2. p53 immunoreactivity and sun-exposure in squamous cell carcinomas

Tumour	Number	Negative (%)	Weakly + ve (%)	Strongly + ve (%)	Total + ve (%)
Squamous cell carcinoma (sun exposed)	18	9 (50)	3 (17)	6 (33)	9 (50)
Squamous cell carcinoma (non sun-exposed)	4	2 (50)	1 (25)	1 (25)	2 (50)

Table 3. p53 immunoreactivity and cellular differentiation in squamous cell carcinoma

Tumour	Number	Negative (%)	Weakly+ve (%)	Strongly+ve (%)	Total+ve (%)
Well-differentiated	11	7 (63)	1 (9)	3 (27)	4 (36)
Moderately differentiated	9	3 (33)	3 (33)	3 (33)	6 (66)
Poorly differentiated	2	1 (50)	0 (0)	1 (50)	1 (50)

(arsenic ingestion in six cases and Gorlin's syndrome in one case) each basal cell carcinoma was excised from sun-exposed skin and consequently the relative contribution of UV-light to tumorigenesis could not be estimated. p53 immunoreactivity was also demonstrated in 80% (4/5) of Bowen's disease, in which UV-light was a probable contributory factor in only one case.

p53 immunoreactivity and squamous cellular differentiation (Table 3)

Squamous cell carcinomas were divided on histopathological criteria into three categories: well-, moderately, or poorly differentiated. Although no significant difference in the prevalence of p53 immunoreactivity was observed between these groups, where positive, strong staining was observed more frequently in poorly differentiated than in well-differentiated tumours (Table 3).

Discussion

This study demonstrates aberrant expression of p53 tumour-suppressor protein in a high proportion of epidermal tumours: 80% of Bowen's disease, 64% of keratoacanthomas, 55% of solar keratoses, 50% of squamous cell carcinomas and 46% of basal cell carcinomas. p53 immunoreactivity was not observed in normal skin or in viral warts. The pattern of p53 immunoreactivity differed between tumours: nuclear staining was observed throughout the thickness of the tumour in Bowen's disease and in basal and squamous cell carcinomas, whereas in solar keratoses and keratoacanthomas, positive staining was confined to the proliferating basal keratinocytes. Although no significant correlation between the prevalence of p53 immunoreactivity and squamous cell differentiation could be demonstrated, strong nuclear staining was more frequently seen in poorly differentiated malignant tumours than in either premalignant or benign tumours. Variable nuclear staining intensity with CM-1 has been described in other tumours,[21,28] including melanoma, where our group and others[29,30] have observed strong p53 immunoreactivity most frequently in invasive nodular melanomas, which are associated with a poor prognosis. The biological significance of this observation is not currently understood. It is possible that heterogeneous p53 immunoreactivity may reflect either altered affinity of CM-1 for different p53 mutants[21] or different levels of cellular p53 protein in different tumours.

p53 immunostaining, in many cases, suggests the presence of a p53 gene mutation. In some instances, however, p53 immunostaining may be found in the absence of a p53 gene mutation, in particular, in the presence of a mutant *ras* oncogene.[31] 'False positive' results such as this, however, may still be biologically important if abnormal stabilization of p53, by other mechanisms, also abolishes its function. Notwithstanding the current uncertainty regarding concordance between p53 immunostaining and p53 gene mutation, our immunohistochemical study, and a recent molecular genetic study,[19] both suggest a prevalence of p53 gene mutation in squamous cell carcinoma of approximately 50%. Our study also demonstrates p53 immunoreactivity in other epidermal tumours, suggesting that p53 gene mutation may be an important step common to many epidermal cancers. Our data contrast with those of a previous study, where p53 immunoreactivity was not found in either basal cell carcinomas or in solar keratoses, and was observed in only 15% of squamous cell carcinomas.[32] Differences may reflect the aetiology of the tumours studied or, possibly, differences in fixation and section preparation techniques.

Epidemiological studies strongly implicate UV-light in the development of many epidermal tumours, including solar keratoses, squamous and basal cell carcinomas.[20,33] The recent demonstration of p53 mutations at dipyrimidine sites and the unique finding of CC to TT double-base alterations in epidermal tumours, but not in other malignancies,[19] provides compelling evidence for a critical role for UV-light in cutaneous squamous cell neoplasia. Our study suggests, however, that other carcinogens involved in cutaneous neoplasia may also result in p53 gene mutation. p53 immunoreactivity was observed, for example, in two of four squamous cell carcinomas in which UV-light was unlikely to be contributory. Similarly, p53 immunoreactivity was observed in a high proportion of Bowen's disease, a tumour in which arsenic ingestion rather than UV-light is thought to play an important aetiological role.[34]

The molecular genetics of epidermal cancers are currently poorly understood, and little is known of the contribution of individual gene mutations to the malignant phenotype of keratinocytes. Current evidence suggests that tumorigenesis proceeds through the stepwise accumulation of genetic alterations.[35] For a given tumour to express a malignant phenotype, the number of mutations appears to be more important than the order in which they are acquired. None the less, specific tumours frequently seem to acquire p53 mutations at a characteristic stage in their evolution. For example, in colorectal[36] and some cases of breast carcinoma,[37] p53 gene mutation appears to be a late event associated with progression of disease, whereas in germ-cell tumours, p53 mutation may occur at the intra-tubular preinvasive stage.[28] Our observation of p53 immunoreactivity in a high proportion of premalignant epidermal tumours, including solar keratoses and Bowen's disease, suggests that p53 gene mutation may similarly occur at an early stage in squamous cell tumorigenesis. Only a small proportion of these premalignant lesions progress to squamous cell carcinomas, however,[34,38] suggesting that further mutations may be required for malignant transformation. Moreover, the demonstration of p53 immunoreactivity in keratoacanthomas, tumours which typically undergo spontaneous regression, suggests that genetic alterations do not necessarily represent irreversible steps in tumorigenesis. In fact, activating mutations in the *H-ras* oncogene, demonstrated in a proportion of keratoacanthomas, have been implicated in the actual process of tumour regression.[39]

This study suggests that p53 gene mutation may play an important role in the development of diverse human skin cancers. In addition, our data support a multistep model for squamous cell tumorigenesis. Although p53 gene mutation may be an early event in this process, occurring in solar keratoses and Bowen's disease, subsequent genetic alterations appear to be required for malignant progression. Molecular analysis of p53 gene mutations occurring in skin tumours of various aetiologies is now required to explore further the relative contribution of UV-light to both melanoma and non-melanoma skin cancer.

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Functional assessment of the stratum corneum under the influence of oral aromatic retinoid (etretinate) in guinea-pigs and humans. Comparison with topical retinoic acid treatment

H.TAGAMI, T.TADAKI, M.OBATA AND J.KOYAMA
Department of Dermatology, Tohoku University School of Medicine, Sendai, Japan

Accepted for publication 1 April 1992

Summary Clinically we have noted that the skin of patients treated with long-term oral etretinate becomes uniformly soft and smooth to touch, like facial skin that becomes smoother and less wrinkled following treatment with topical tretinoin. This suggests that retinoids, whether used systemically or topically, alter the physical properties of the skin, particularly of the stratum corneum (SC). To study the influence of retinoids on the SC, we serially assessed the functional properties of the SC non-invasively in retinoid-treated humans and experimental animals. SC hydration and barrier function were assessed by measurement of high-frequency conductance and transepidermal water loss (TEWL), respectively. Daily application of topical retinoic acid creams was found to rapidly induce a time- and dose-dependent, linear increase in SC hydration of the forearm skin of healthy adults over a 2-week period and to compromise its water barrier function in a similar fashion. Systemic administration of high-dosage etretinate, 4 or 8 mg/kg/day, to guinea-pigs also induced dose-dependent increases in both SC hydration and TEWL measured on the plantar skin after 1 month. Moreover, in the animals given etretinate 4 mg/kg/day we confirmed a slight but significant decrease in the number of cell layers of the plantar SC. Likewise, patients with various dermatoses began to show similar functional changes of the SC in the uninvolved skin of the flexor surface of the forearms 3 weeks after the start of oral etretinate treatment, consisting of 50 mg daily for 2 weeks, followed by gradual dose tapering. Thus, it is reasonable to speculate that oral etretinate changes the properties of the SC to produce smoother skin over the whole body surface, probably by increasing amounts of water-binding substances in the SC as well as by exerting a keratolytic effect, in a fashion similar to that induced by topical tretinoin application.

Post-pubertal patients with acne describe smoother, less wrinkled skin after treatment with topical tretinoin. Such observations prompted Kligman *et al.*[1] to study the effect of tretinoin on photoaged skin. A synthetic vitamin A derivative, etretinate, has been widely used for the treatment of disorders of keratinization.[2,3] Despite numerous reports of the dramatic improvement of various skin disorders, the changes that occur in normal skin after the intake of etretinate have been overlooked or neglected, except for side-effects such as palmoplantar desquamation and cheilitis. We have noticed that, in addition to the improvement of lesional skin, the uninvolved skin of these patients also undergoes concomitant change. The skin surface becomes uniformly soft and smooth to touch, and shiny pink in appearance. These changes return to a pretreatment state after cessation of therapy. The changes resemble those seen in skin treated with topical tretinoin.[1]

Changes induced by topical tretinoin, such as smoothness and softness, are properties principally determined by changes induced in the stratum corneum (SC), whereas decreased wrinkling may involve alterations induced in components of the dermis, although the precise underlying mechanisms are still unknown. The softness and flexibility of the skin also depend to some extent on the properties of the connective tissue, and Berardesca *et al.*[4] reported that topical treatment with tretinoin increased skin elasticity. However, SC hydration has a much more direct and greater influence on the suppleness and smoothness of the skin, because it is

Correspondence: Dr H.Tagami, Department of Dermatology, Tohoku University School of Medicine, 1-1 Seiryomachi, Aobaku, Sendai 980, Japan.

the water contained in the superficial SC which keeps the skin surface supple and flexible.[5] Retinoids are known to exert profound influences on epithelial differentiation.[6] Systemic administration of either etretinate or isotretinoin was reported to induce impairment of epidermal barrier function manifest by increased transepidermal water loss (TEWL).[7,8] However, to our knowledge, there have been no studies of the effects of oral retinoids on another important function of the SC, its water-holding capacity. SC hydration can be evaluated non-invasively by measuring cutaneous conductance against high-frequency current.[9] It closely correlates with the actual water content in the superficial portion of the SC as well as that in the whole SC.[10] In this study, utilizing non-invasive *in vivo* techniques, we have evaluated the functional properties of the SC induced by prolonged oral intake of etretinate, in comparison with those induced in human skin by topical tretinoin application.

Methods

Animals

Ten female albino guinea-pigs (Hartley strain), each weighing 200 g, were used. Five animals were given etretinate 4 mg/kg as a single daily dose for 4 weeks by oral intubation. Etretinate was dissolved in 200 μl of peanut oil. The remaining five animals, which served as controls, were given only 200 μl of peanut oil daily by oral intubation. Glabrous skin on the plantar surface of a hind foot was chosen as a test site for skin surface hydration state and TEWL measurements because no hair-plucking was required at this site. After 4 weeks, a biopsy specimen was obtained from the test site and immediately frozen in liquid nitrogen.

Human subjects

For the evaluation of the effects of topical tretinoin treatment 0·02 ml of 0·025, 0·05 and 0·1% retinoic acid creams and their base (Retin-A® creams and the cream base were gifts from Ortho) were applied once daily to four square skin areas, respectively, which were demarcated with a 4-cm margin on the flexor surfaces of the forearms of 10 healthy adult male volunteers aged 30 ± 2 years.

Five male and seven female patients with a mean age of 48 (range 27–60) years participated in the study for the evaluation of oral etretinate. They comprised four patients with psoriasis, two with palmoplantar pustulosis, and one each with the following disorders: pustular psoriasis, psoriatic arthritis, pityriasis rubra pilaris, palmoplantar hyperkeratosis, subcorneal pustular dermatosis and lichen sclerosus et atrophicus. Oral administration of etretinate was started with a dose of 50 mg (approximately 1 mg/kg/day) and this was continued for 2 weeks. Thereafter, the dose was gradually tapered at a rate of 5 mg/week until a minimum effective maintenance dose of 15–20 mg/day was reached. Measurements of 3·5 MHz conductance and TEWL were performed on uninvolved skin on the flexor surface of the forearm immediately before etretinate therapy and 1, 3, 5, 7 and 8 weeks after the start of treatment. Because it was difficult to obtain age- and sex-matched controls at each evaluation time, as a control we used the same 24-year-old female technician, on whose skin, using the same site on the flexor surface of the left forearm, the same measurements were performed on each occasion.

The measurements were performed in various seasons of the year, but were made at room temperature of 18–22°C and relative humidity of 20–64% to avoid non-physiological conditions and the influence of sweating.

Skin-surface hydration state

Electrical conductance measurements were made using a skin-surface hygrometer (Skicon-200, IBS Co, Hamamatsu, Japan) as reported previously.[10] The measurements were repeated at least three times to obtain an average.

Transepidermal water loss

The TEWL was measured using an Evaporimeter (Servo Med AB, Vallingby, Sweden), as described by Nilsson.[11] In the guinea-pigs, it was impossible to obtain accurate readings on their plantar skin surface which was too small for the cylindrical probe of the Evaporimeter. We therefore measured water evaporation through a round hole 1 cm in diameter made in a 1-mm thick plastic board which was used to press the plantar surface flat.

Determination of the number of cell layers in the stratum corneum

Cryostat sections (5 μm) prepared from the frozen specimens of plantar skin from guinea pigs, which had received etretinate 4 mg/kg/day for 4 weeks, were swollen with 1 N NaOH, stained with fluorescent isothionate, and the number of cell layers counted under a fluorescence microscope.

Statistical analysis

The data were expressed as the mean ± standard deviation. Student's *t*-test was used to analyse data for statistical significance ($P < 0{\cdot}05$).

Results

Topical retinoic acid application

Topical 0·1% retinoic acid cream applied to the flexor surface of the forearms of 10 healthy adults generated faint erythema which persisted for 2 days without desquamation in three of 10 subjects from 5 to 7 days after the start of daily application. The retinoic acid creams induced a linear, dose- and time-dependent increase in both high-frequency conductance and TEWL over a 14-day treatment period (Figs 1 and 2).

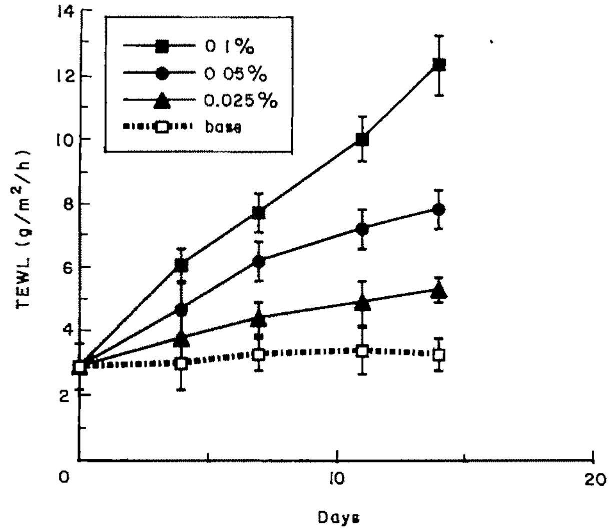

Figure 2. Transepidermal water loss (TEWL) measured on the flexor aspect of the forearm in 10 volunteers treated with various retinoic acid creams and a cream base. Data are presented as means ± SD.

Systemic etretinate administration

(i) Studies in guinea-pigs. Etretinate intake at a dose of 4 mg/kg/day induced slight erythematosquamous changes on the eyelids and in the perioral region within 1 week. There was no plantar desquamation. However, high-frequency conductance values on the plantar skin began to increase 1 week after starting daily intake of etretinate, attaining a level which was statistically significant compared with that of the controls after 3 weeks ($P < 0{\cdot}01$) [Fig. 3]. In contrast, a significant increase in TEWL did not occur until 4 weeks in these animals ($P < 0{\cdot}01$) [Fig. 4]. The number of cell layers of the SC at this point was 67 ± 5 in the etretinate group, which was significantly less than the value of 72 ± 6 in the control group ($P < 0{\cdot}05$).

We doubled the daily dose of etretinate to 8 mg/kg in another group of five animals to assess whether there was any dose–response relationship, and conducted functional measurements at the end of a 4-week treat-

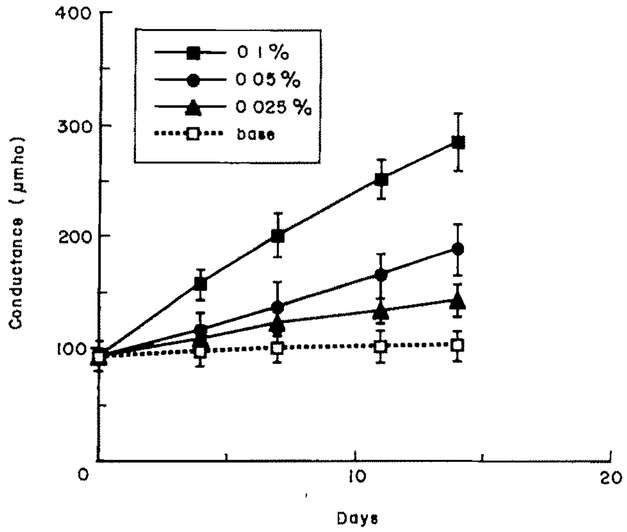

Figure 1. Conductance for high-frequency current measured on the flexor surfaces of the forearms in 10 volunteers treated with various retinoic acid creams or a cream base. Data are presented as means ± SD.

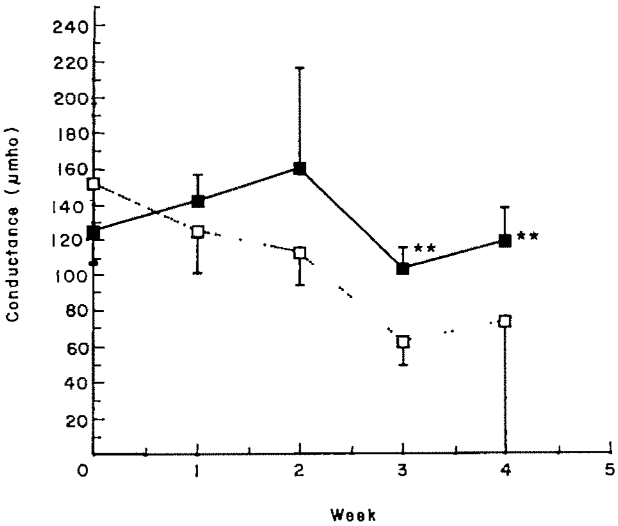

Figure 3: Conductance for high frequency current measured on the plantar skin of guinea-pigs given oral etretinate (4 mg/kg/day) (■). Conductance measured in control animals (□). Data are presented as means ± SD. ** $P < 0{\cdot}01$.

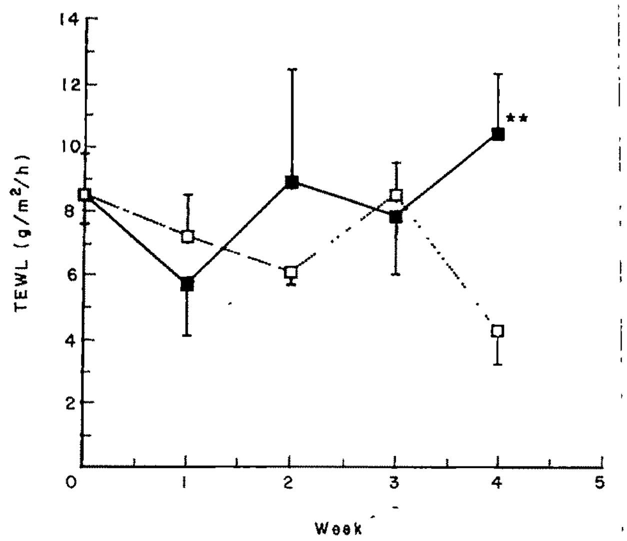

Figure 4: Transepidermal water loss (TEWL) measured on the plantar skin of guinea-pigs treated with oral etretinate (4 mg/kg/day) (■). TEWL measured in control animals (□). Data are presented as means±SD. ** $P<0{\cdot}001$.

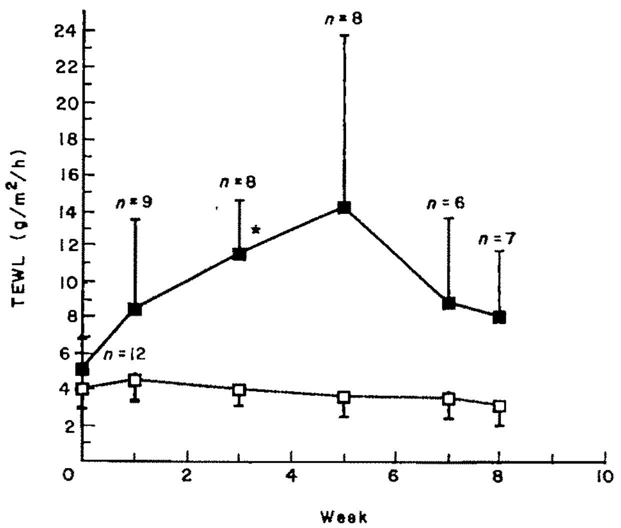

Figure 6. Transepidermal water loss (TEWL) measured on the flexor aspect of the forearm in patients treated with oral etretinate (■). TEWL values in a normal control (□). * TEWL values are statistically significantly different ($P<0{\cdot}05$) from controls for all time points beyond asterisk.

ment period. In this case, the differences between etretinate-treated animals and controls were more prominent than those in animals treated with 4 mg/kg/day (conductance 154·3±15·9 μmho vs. 93·0±6·1 μmho in controls ($P<0{\cdot}001$); TEWL 8·9±0·7 g/m²/h vs. 5·9±0·6 g/m²/h in controls ($P<0{\cdot}001$).

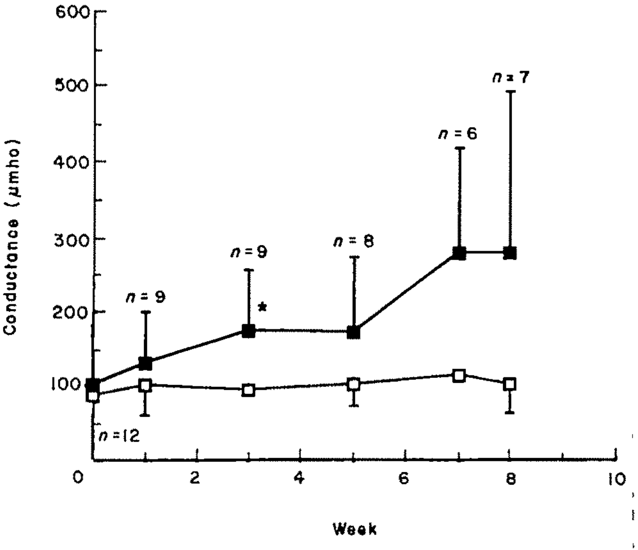

Figure 5. Conductance for high-frequency current measured on the flexor surface of the forearm in patients treated with oral etretinate (■). * Conductance values are statistically significantly different ($P<0{\cdot}05$) from controls (□) for all time points beyond asterisk.

(ii) Studies in patients. Within 1 week of the start of etretinate therapy, all patients complained of a dry mouth and desquamation of the lips and palmoplantar skin, which lessened after tapering of the initial high doses of etretinate. At the same time the patients' uninvolved skin began to feel soft and smooth to touch, and became pink in colour.

Non-invasive functional measurements of the SC showed that high-frequency conductance began to increase 1 week after the start of therapy, attaining a significantly higher level after 3 weeks, and continued to increase gradually until 8 weeks (Fig. 5). TEWL also showed an increase after initiation of treatment, reaching a significantly higher level after 3 weeks. It reached a peak after 5 weeks, and thereafter gradually decreased, but maintained significantly higher levels even after 8 weeks (Fig. 6).

Discussion

It is reasonable to assume that high cutaneous retinoid concentrations can be attained more rapidly by topical rather than systemic administration. Indeed, the present results show that even short-term treatment of human skin with topical tretinoin increases SC hydration, with a concomitant decrease in its water barrier function demonstrable as elevated TEWL, in a dose-dependent fashion. Such a rapid increase in SC hydration appears to correlate well with the recent histological findings of Fisher *et al.*[12] who demonstrated increased mucin deposition, which is induced by increased transforming growth factor-β, after 4-day treatment of human skin with 0·05% tretinoin cream under occlusion. Moreover, the increased SC hydration and elevated TEWL are likely to be a constant feature in the retinoid-treated skin, because the skin, regardless of photoageing, is reported to show these changes after long-term topical application of tretinoin.[1,13]

Elias *et al.*[7] reported that in rats oral etretinate induced an increase in TEWL, which reflects a reduction in the water barrier function of the SC. However, no functional assessment of the water-holding capacity of the SC, which plays a crucial role in maintaining the softness and smoothness of the skin surface, has been performed either in experimental animals or in humans. The functional assessment of the SC in this present study showed that the usual long-term therapeutic regimen of oral etretinate, like topical retinoic acid treatment, induces not only an elevation in water evaporation from the skin but also an increase in high-frequency conductance, a parameter for SC hydration, of the uninvolved skin of patients with various dermatoses.

Functional changes of the SC under the influence of oral etretinate were also detected in guinea-pigs, and we showed that they occur in a dose-dependent fashion. For technical reasons, we chose the hairless plantar skin of the animals for assessment of SC function to avoid the influence of hair-plucking. Because their plantar skin surfaces were not large enough to occupy the whole area of the probe of the Evaporimeter, we compared relative changes of TEWL based on the measurements of evaporated water lost through a hole in a plastic board used to press the plantar skin flat. We think that similar, probably more pronounced, changes might have been demonstrated if we had used the back skin of hairless guinea-pigs.

In contrast with the animal experiments, which could be conducted in a uniform way in the same season, we were obliged to perform the functional assessment in human subjects on different occasions between each subject, because, instead of healthy volunteers, we used patients who were being treated with oral etretinate for their skin diseases. Moreover, due to the difficulty of recruiting an age-matched volunteer for each patient, we used the skin of the flexor surface of the forearm of the same 24-year-old female technician as a control each time. Thus, there were much larger inter-individual variations in the data obtained from the patients than from the control.

In our previous study[14] we found that even partial removal of the superficial layers of the SC by adhesive tape stripping induces a remarkable increase in the skin surface hydration level. A decrease in the SC cell layers not only facilitates water passage through the SC but also exposes more hydrated deep-seated SC on its surface.[15] Etretinate in a high dose (25 mg/kg/day) was found to exert a keratolytic, cytotoxic and mucoplastic effect on normal guinea-pig epidermis.[16] Premature desquamation of cultured keratinocytes from human foreskin epidermis has also been reported on exposure to retinoic acid.[17] The results of our present morphometrical analysis of the SC of the plantar skin of guinea-pigs also provides evidence indicating that orally administered etretinate, although administered in much smaller dosage than in previous studies, exerts a mild keratolytic effect, to decrease the number of layers of corneocytes in the SC. However, we were unable to perform similar studies in human subjects, because it was not possible to obtain consent from the patients with various dermatoses to at least two biopsies from their non-lesional skin, before and during etretinate therapy.

The exposure of better hydrated, deep-seated portions of the SC by the keratolytic effect of etretinate is thought to induce an increase in suppleness and smoothness of the skin surface even in extremely dry ambient conditions. Nevertheless, the extent of the decrease in SC cell layers counted in the plantar skin of the guinea-pigs treated with an extremely high dosage of etretinate appeared too small to explain the changes observed in functional parameters. Elias *et al.*[7] and Elias[18] ascribed the increase in TEWL noted in etretinate-treated animals to loosening of the SC due to loss of desmosomes, and to intra- and inter-cellular accumulation of amorphous material in the upper epidermis. The changes in human skin observed during prolonged administration in ordinary therapeutic doses would be much milder than those noted in experimental animals receiving extremely high doses of etretinate; isotretinoin and etretinate administered for the treatment of various dermatoses were reported to induce only epidermal acanthosis without any statistically significant increase in epidermal DNA synthesis.[19] We think that such effects of etretinate on the epidermal differentiation process, including the intra- and inter-cellular accumulation of amorphous material (glycoconjugates), play an important role in generating the characteristic clinical and functional changes.[18,20] The clinical cutaneous side-effects of etretinate, such as cheilitis and desquamation of palmoplantar skin, appear to be changes induced by mechanisms similar to those described above; they merely occur at sites specifically vulnerable to the effects of etretinate.

In conclusion, retinoids increase the suppleness and smoothness of the skin by affecting the water content of the stratum corneum.

Acknowledgment

The present study was partly supported by a Grants-in-Aid No. 02557044 from the Ministry of Education, Science and Culture, Japan.

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British Journal of Dermatology (1992) 127, 476–481.

Humoral responses to *Malassezia furfur* serovars A, B and C in normal individuals of various ages

A.C.CUNNINGHAM,* E.INGHAM AND G.GOWLAND

The Academic Unit of Immunology, Department of Clinical Medicine, The Old Medical School, University of Leeds, Leeds LS1 3EX, U.K.

Accepted for publication 1 June 1992

Summary

A transferable solid-phase (TSP) ELISA was developed for the determination of antibody titres specific to *Malassezia furfur* serovars A, B and C in human sera. A survey of levels of class-specific antibodies (IgM, IgG and IgA) to *M. furfur* serovars A, B and C in relation to age (2–64 years; 60 individuals) demonstrated that individuals had immunity to *M. furfur* by the age of 2–3 years. There was no difference in either IgM or IgG levels into adulthood. The only age-related differences were lower IgM titres to the three serovars in the 60–64 year age-group compared with younger individuals. There was, however, a difference between titres of antibody specific to the three serovars. The mean reciprocal $\log_2$ IgM titre to serovar A (6·9) was significantly higher ($P<0{\cdot}05$) than that to serovar B (mean reciprocal $\log_2$ titre of 5·8), but not to serovar C (6·1). In contrast, the mean reciprocal $\log_2$ IgG titre to serovar A (6·5) was significantly lower ($P<0{\cdot}05$) than those to serovars B and C (mean reciprocal $\log_2$ titre of 8·9 in both cases).

Malassezia furfur is a dimorphic, lipophilic yeast normally resident on human skin in the sebum-rich areas of the upper trunk and head.[1–3] In its yeast phase, *M. furfur* can be classified into three serovars (A, B and C) based on serological differences in cell-surface antigens.[4] Microscopically, serovars A and B have round blastospores, whereas serovar C possesses oval blastospores. Historically, these may have been described as *Pityrosporum orbiculare* and *P.ovale*, respectively.

There is relatively little information concerning the carriage and distribution of the three serovars of *M. furfur* on the skin of normal individuals at various ages. It has recently been shown that serovar A predominates on the trunk, whilst serovar C is more prevalent on the head, of normal adults.[5] Investigations of *M. furfur* colonization in children have generated conflicting reports. It has been estimated that over 70% of children harbour *M. furfur* on the scalp.[6,7] In contrast, colonization of the upper trunk in children was found to be between 0 and 10%.[8,9]

Antibodies specific to *M. furfur* have been detected in the sera of normal individuals and those suffering from *M. furfur*-associated dermatoses.[10–12] It has been reported that children and elderly individuals have lower antibody levels to *M. furfur* whole cells (serovar A) than young and middle-aged adults.[13,14] Sohnle *et al.*[15] failed to find any differences in IgG titres specific to a homogenized extract of *M. furfur* (a round variant) between a group of young adults and elderly individuals. The levels of IgM were, however, lower in the elderly individuals. Low levels of *M. furfur*-specific IgA were present in both groups.

It has been demonstrated that both the level and class of *Propionibacterium acnes*-specific antibodies in the sera of normal individuals mirror its carriage.[16,17] The distribution of this normal member of the cutaneous flora appears to be similar to that of *M. furfur*, that is, predominantly in regions with a high sebum excretion rate.[3,18] High titres of IgM agglutinins specific to *P. acnes* were detected in children (mean reciprocal $\log_2$ titre 10), whereas lower levels were present in adults (mean reciprocal $\log_2$ titre 8). The IgG titres were low throughout childhood (mean reciprocal $\log_2$ titre 5–6), but dramatically increased in adults (mean reciprocal $\log_2$ titre 11–12). The class of antibody recognizing *P. acnes* therefore changed from IgM to IgG between the ages of 14 and 18. This represented a change from a primary to a secondary host immune response to *P. acnes* and correlated with the increased colonization of individuals by *P. acnes* at puberty.

In this study, a novel transferable solid-phase (TSP) ELISA was developed to measure the levels of class-

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Correspondence to Dr E.Ingham.

*Present address: Department of Surgery, The Medical School, The University, Newcastle upon Tyne, NE2 4HH specific antibodies (IgM, IgG and IgA) to *M. furfur* serovars A, B and C in the normal population at various ages (2–64 years).

Methods

Subjects

Serum samples obtained from 50 females were used in the study (five age groups, each comprising 10 individuals; 2–3, 7–10, 20–24, 33–40 and 60–64 years of age). The children's sera were kindly provided by Dr Alan Balfour, Toxoplasmosis Unit, Regional Public Health Laboratory, Leeds. Adult volunteers were not atopic and had no history of dermatoses (as determined by a questionnaire). Sera from an additional 10 individuals (aged 14–26 years; three female, seven male) with severe acne ($n = 8$) or seborrhoeic dermatitis ($n = 2$) were also included. Test sera were coded and analysed blind for class-specific antibodies (IgM and IgG) to *M. furfur* serovars A, B and C. Thirty-six of the test sera (7–10, 20–24, 60–64 years, plus individuals with dermatoses, nine per group) were also analysed blind for IgA levels to *M. furfur* serovars A, B and C.

Yeast strains

Details of the three strains of *M. furfur* used, representative of serovars A, B and C (strain numbers 13, 5 and 7, respectively) have been described previously.[4] Samples were originally obtained from sebum-rich areas of clinically normal skin by application of a cotton wool swab moistened with 'wash fluid' (phosphate-buffered 0·1% Triton X-100 solution, pH 7·9). These were immersed in 1 ml of half-strength wash fluid and appropriate dilutions incubated on a selective medium[19] in a moist environment at 34°C for 14 days. From 132 strains isolated, three morphologically stable, serologically distinct variants were defined.

Logarithmic-phase organisms were used as the antigen in the ELISA. A heavy inoculum of the appropriate strain of yeast cells was transferred to 10 ml of liquid medium (containing 0·1 g/l glucose [Sigma]; 10 g/l peptone [Oxoid]; 10 g/l yeast extract [Oxoid]; 2 g/l sodium taurocholate [BDH]; 0·4 ml/l ultra-high-temperature treated milk [Associated Dairies, Leeds]; pH 6·2). Chloramphenicol (50 mg/l) and cycloheximide (200 mg/l) were added after autoclaving, and incubated at 34°C, with shaking. When turbid, these starter cultures were used to inoculate 100 ml of liquid medium which was incubated in the same way. When the cultures attained the appropriate optical density (600 nm) the yeast cells were harvested by centrifugation (750 *g* for 15 min), resuspended in phosphate-buffered saline (PBS; Dulbecco B, Oxoid) on a magnetic stirrer at 4°C, and used within 12 h.

Determination of antibody levels to M. furfur serovars A, B and C

A reproducible, transferable solid-phase (TSP) ELISA was developed to measure class-specific antibody titres to *M. furfur* serovars A, B and C.[20] This assay was similar to conventional ELISA systems, except that the yeast cells were attached to plastic pins which were transferred between reagents in microtitre plates, facilitating the washing process. Logarithmic phase *M. furfur* whole cells, representative of each of the three serovars, were coated on to the TSP (Nunc) by immersion in sterile round-bottomed microtitre (MT) plates (Nunc) containing 150 μl of 3×10^8 cells/ml in coating buffer (0·015 M Na_2CO_3; 0·035 M $NaHCO_3$; 0·02% NaN_3 in 1l deionized water, pH 9·6) for 2·5 h at room temperature (RT), with shaking (Hybriturn 9630001). The yeast-coated TSP were blocked by immersion in wash buffer (PBS containing 0·05% v/v Tween 20 [BDH] and 1% w/v bovine serum albumin [Sigma] pH 7·4) for 5 min, air-dried for 30 min and vacuum-packed in a plastic bag with a desiccant. These were usable for up to 2 weeks when stored at 4°C. The TSP were re-wetted with wash buffer for 5 min just before use. For the assay, the yeast-coated TSP were immersed in round-bottomed MT plates (Nunc) containing 200 μl of the appropriate serum dilutions in duplicate for 2 h at RT. The TSP were washed by immersion in wash buffer (2×5 min) and placed in another round-bottomed MT plate containing the appropriate optimum dilution of alkaline phosphatase-conjugated, affinity-purified, goat anti-human immunoglobulins (Seralab, specific for the heavy chains of IgM, IgG and IgA, respectively) for 3 h at RT. After washing, the TSP were then submerged in flat-bottomed MT plates containing 200 μl of freshly prepared *p*-nitrophenyl phosphate (Sigma; 1 g/l in 10% diethanolamine buffer containing 9·7% diethanolamine [BDH]; 0·01% $MgCl_2.6H_2O$ [BDH]; 0·02% NaN_3 [BDH]; pH 9·8) until internal positive control wells had reached a predetermined optical density. The reaction was stopped by the addition of 50 μl of 3 M NaOH and absorbance values read at a wavelength of 405 nm with an ELISA plate reader (Lab Systems Uniskan 1). Relevant controls were included on each plate. The end-point of the assay was reached when replicate positive control wells reached a predetermined absorbance value (a batch of pooled AB serum, obtained from the NHS blood transfusion service, which had been titrated for IgM and IgG levels specific to *M. furfur* serovars A, B and C). The IgA positive control was a batch of pooled sera collected from patients with severe acne, because IgA levels in the pooled AB serum were low, thereby enabling the direct comparison of individual plates. The antibody titres of the test sera were taken as the reciprocal $\log_2$ value + 1 of the last dilution with an absorbance value of greater than or equal to twice the negative control serum (positive control serum successively absorbed with formalized logarithmic-phase *M. furfur* cells of strains 13 [serovar A], 5 [serovar B] and 7[serovar C]) at the same dilution.

Statistical analyses

The class-specific antibody titres to *M. furfur* serovars A, B and C in the six groups of individuals were analysed by two-way analysis of variance. The significance of any differences between age groups was tested by the method of minimum significant differences (MSD) for multiple unplanned comparisons between means.[21]

Results

IgM levels to M. furfur serovars A, B and C

The two-way analysis of variance revealed a significant variation between both age-groups ($P<0\cdot01$) and serovars ($P<0\cdot01$). Individuals had measurable IgM antibody titres specific to *M. furfur* serovars A, B and C by the age of 2–3 years (mean $\log_2+1$ titre of 7·1). These were comparable with adult values (Fig. 1). However, specific IgM titres were significantly lower in the 60–64 year age-group. Antibody levels to serovar A were significantly lower ($P<0\cdot05$) than those in the 2–3, 7–10 and 33–40 year age-groups; antibody titres to serovar B were significantly lower ($P<0\cdot05$) than those in the 2–3, 7–10 and 20–24 year age-groups. Mean antibody titres to *M. furfur* serovar A were higher than those to B and C in each group. When the results were analysed for differences between serovars only, IgM levels specific to serovar A were significantly higher ($P<0\cdot05$) than those to serovar B (Fig. 2).

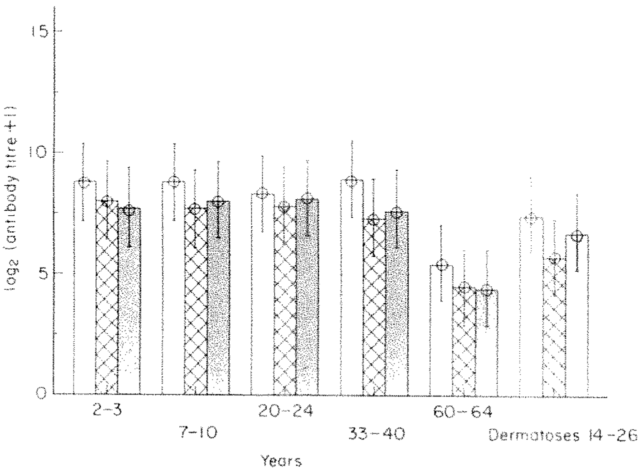

Figure 1. Mean IgM titres to serovars A □, B ▨ and C ▩ in individuals of varying ages. The results are the mean of duplicate determinations of 10 serum samples per age group and are expressed as the mean ± ½ the MSD (±1·6; $P<0\cdot05$).

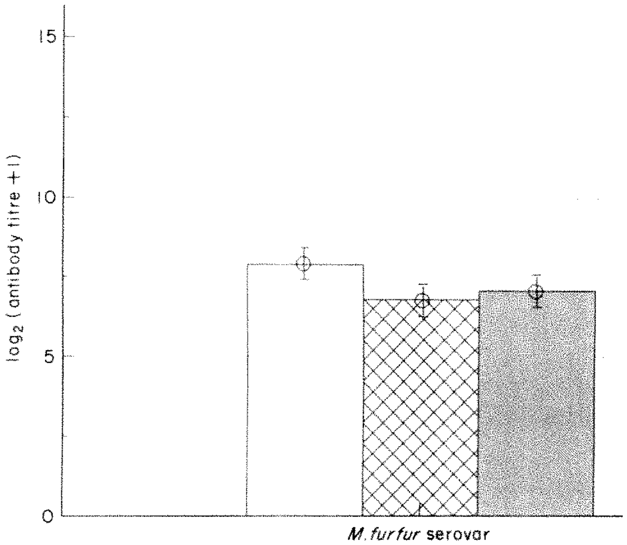

Figure 2. Mean IgM titres to serovars A □, B ▨ and C ▩. The results are the mean of duplicate determinations of 60 serum samples and are expressed as the mean ± ½ the MSD (±0·5; $P<0\cdot05$).

IgG levels to M. furfur serovars A, B and C

Two-way analysis of variance revealed a significant variation amongst age-groups and serovars ($P<0\cdot01$). The means were compared by the MSD method, and there were no significant differences ($P>0\cdot05$) in antibody titres in individuals aged between 2 and 64 years (Fig. 3; mean $\log_2+1$ titre of 9·3 in the 2–3 year age-group). However, there was a large degree of individual variation in the IgG titres to *M. furfur*, as indicated by the relatively large error bars. Mean antibody titres to serovar A were lower than those to B and C in each group. When the data was reanalysed for serovars only, it was clear that IgG titres to serovar A were significantly

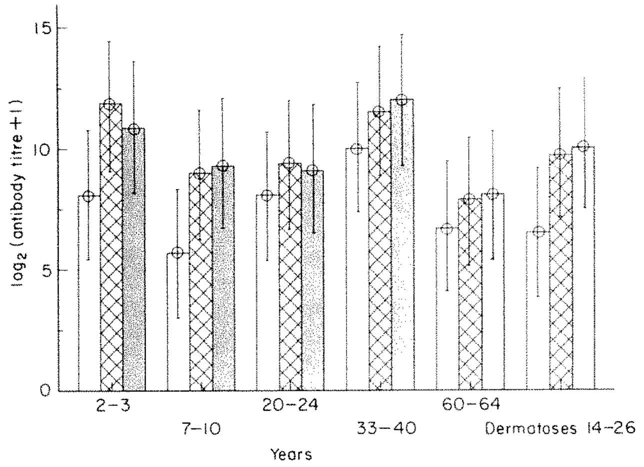

Figure 3. Mean IgG titres to serovars A □, B ▨ and C ▩ in individuals of varying ages. The results are the mean of duplicate determinations of 10 serum samples per age group and are expressed as the mean ± ½ the MSD (±2·7; $P<0·05$).

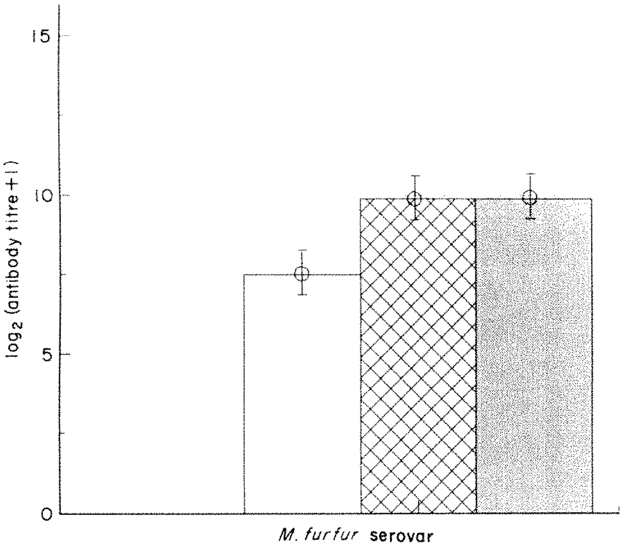

Figure 4. Mean IgG titres to serovars A □, B ▨ and C □. The results are the mean of duplicate determinations of 60 serum samples and are expressed as the mean ± ½ the MSD (±0·7; $P<0·05$).

lower than those to B and C ($P<0·05$) in the 60 sera tested (Fig. 4).

IgA levels to M. furfur A, B and C

IgA titres were undetectable in 18 of the sera tested, and were generally very low. There was no significant difference ($P>0·05$) between either the serovars or the groups (Fig. 5).

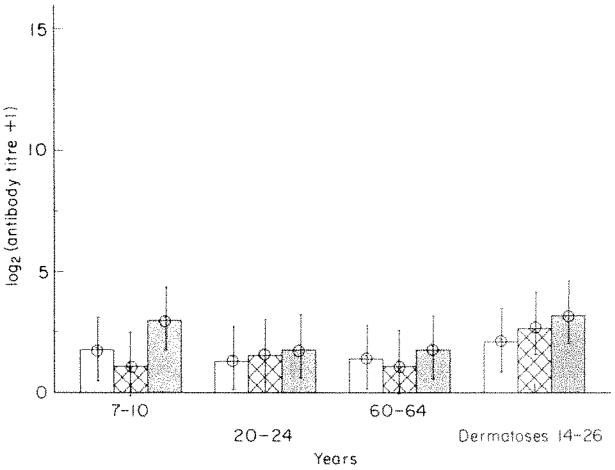

Figure 5. Mean IgA titres to serovars A □, B ▨ and C ▩ in individuals of varying ages. The results are the mean of duplicate determinations of nine serum samples per age group and are expressed as the mean ± ½ the MSD (±1·3; $P<0·05$).

Discussion

This study has demonstrated that individuals possessed both IgM and IgG antibodies reactive with *M. furfur* serovars A, B and C by the age of 2–3 years (mean reciprocal $\log_2 + 1$ titres of 7·1 for IgM and 9·3 for IgG). These antibody levels were comparable with adult values. The small sample of individuals with dermatoses showed no significant differences in antibody levels to *M. furfur* compared with the rest of the population. The only age-related difference was a reduction in IgM levels in the elderly group (60–64 years; mean reciprocal $\log_2 + 1$ titre 4·8). It is possible that this reflected less stimulation by *M. furfur*, because it has been reported that elderly individuals harbour lower numbers of *M. furfur* than young adults.[14] It is also conceivable that the immune system of elderly individuals is less able to respond to primary stimulation by *M. furfur*. Bergbrant and Faergemann[14] reported a reduction in IgG titres to *M. furfur* serovar A with increasing age. However, the less sensitive technique of indirect immunofluorescence was used in that study. Sohnle *et al.*[15] failed to find a difference in IgG levels to *M. furfur* by ELISA in a group of elderly individuals (70–88 years) compared with younger adults (23–40 years). There were, however, lower IgM levels in the elderly group. In comparison, no difference was demonstrated in IgM antibody titres to *Candida albicans* and *Trichophyton rubrum* in the elderly group.

It has been suggested that *M. furfur* colonization increases at puberty as a result of the changes in sebaceous gland activity. Leyden *et al.*[18] demonstrated that this occurs with the lipophilic bacterium, *P. acnes*. If it also occurs with *M. furfur*, humoral immunity might be expected to demonstrate a similar trend to *P. acnes* humoral immunity. Faergemann's[13] observation of lower antibody levels to *M. furfur* in children (up to the age of 15 years), compared with adults, would tend to support this hypothesis. In this study, both IgM and IgG antibody levels specific to *M. furfur* serovars A, B and C remained relatively constant between 2 and 40 years, indicating that a distinct change from a primary response (IgM) to a secondary response (IgG) did not occur at puberty. This is clearly different from the humoral response to *P. acnes* In contrast, *M. furfur* appears to stimulate the immune system from at least 2 years of age.

Levels of IgA to *M. furfur* serovars A, B and C were very low or undetectable in normal individuals of all ages tested. This result was in agreement with Sohnle *et al.*[15] who only measured IgA titres in sera which had been diluted 1:5.

It was an interesting observation that mean IgM antibody titres to *M. furfur* serovar A (mean reciprocal $\log_2+1$ titre of 6·9) were higher than those to serovars B and C (mean reciprocal $\log_2+1$ titre 5·8 and 6·1, respectively), whereas IgG antibody titres were lower (mean reciprocal $\log_2$ titre+1 6·5 as opposed to 8·9 for serovars B and C). This may reflect a difference in the stimulation of the host immune system by the three serovars of *M. furfur*; the host response to serovar A may be of a more primary nature than that to serovars B and C. *M. furfur* serovars A, B and C possess common antigenic determinants in addition to their group-specific antigens,[4] and this cross-reactivity may be reflected in the similarity between antibody levels to serovars B and C. Preliminary findings indicate that the three serovars have a different distribution on the body,[5] and as such they may be presented to the immune system by different routes. It therefore appears that there may be some physiological significance to the fact that *M. furfur* can be differentiated into three distinct serovars on the basis of yeast-phase cell-surface antigens.

M. furfur is a normal skin saprophyte, but it is increasingly implicated as an opportunistic pathogen in a range of dermatoses including pityriasis versicolor,[22] seborrhoeic dermatitis and dandruff,[23] folliculitis,[24] atopic dermatitis[25] and fungaemia in immunocompromized individuals receiving parenteral intralipid.[26] It is thought that the host response to this organism is a significant factor in the onset of disease.[27] An increased understanding of man's normal immunity to *M. furfur* serovars A, B and C is therefore essential, so that any differences in immune responses in disease states can be evaluated.

Acknowledgment

The authors thank Richardson-Vicks Ltd for financial support.

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British Journal of Dermatology (1992) 127, 482–484.

Variation in the deposition of the antibodies at different anatomical sites in linear IgA disease of adults and chronic bullous disease of childhood

P.M.COLLIER,* F.WOJNAROWSKA* AND P.R.MILLARD†

**Department of Dermatology, Slade Hospital, Oxford, U.K.*

†Department of Histopathology, John Radcliffe Hospital, Oxford, U.K.

Accepted for publication 6 April 1992

Summary

The diagnosis of linear IgA disease of adults (LAD) and chronic bullous disease of childhood (CBDC) relies upon finding a linear band of IgA at the basement membrane zone on direct immunofluorescence. This study examines the regional variation in antigen expression in the skin of affected individuals. Direct immunofluorescence was performed on biopsies from four different sites in 17 patients with these diseases. In two patients a biopsy from the volar surface of the forearm was negative, but other sites were positive; in the remaining patients there was no variation in antibody expression with site. It is therefore recommended that, if a single diagnostic biopsy is to be taken, the volar surface of the forearm is avoided.

Linear IgA disease of adults (LAD) and chronic bullous disease of childhood (CBDC) are dapsone-responsive autoimmune subepidermal blistering diseases characterized by linear IgA deposits at the basement membrane zone, and often by circulating IgA antibodies to the basement membrane zone. The target antigen has recently been identified, and shown to be identical in both LAD and CBDC.[1]

The linear IgA antigen has been found in all mammalian stratified squamous epithelia studied.[2] Semi-quantitative studies comparing the concentrations of linear IgA antigen in normal skin, oral mucosa and in conjunctiva have shown that, although present in all three tissues, it is in lower concentrations in conjunctiva.[3] However, a study comparing the deposition of the antibodies in normal, clinically uninvolved skin, with the deposition in oral mucosa and conjunctiva, demonstrated IgA deposits in the skin and oral mucosa, but not in conjunctiva.[4] There have been no studies investigation the regional variation in the deposition of IgA antibodies in LAD and CBDC.

Studies on patients with pemphigus have shown no regional variation in antibody deposition,[5] despite variation in expression of the pemphigus antigens.[6] In patients with generalized bullous pemphigoid, Weigand and Clements[7] did not find any variation in deposition of antibody between specific anatomical sites in biopsies from perilesional skin. However, Anstey *et al.*[8] reported regional variation in positive immunofluorescence in clinically uninvolved skin, with the lower back being most frequently negative on direct immunofluorescence in a pretreatment group. Venning *et al.*[9] demonstrated that mucosal sites (oral mucosa and conjunctiva) are positive more often than clinically uninvolved skin in bullous pemphigoid. The bullous pemphigoid antigen shows quantitative variation in its expression and this correlates with the clinical pattern of the disease rather than with the antibody deposition.[10]

This study addresses the question of the variation of *in vivo* deposition of the anti-basement membrane zone antibodies in LAD and CBDC with body site. This knowledge is of practical relevance in determining the optimum site for diagnostic biopsies, in addition to extending our understanding of this condition.

Patients and methods

Sixteen patients with LAD and one with CBDC were studied. The clinical details are summarized in Table 1. Remission was defined as no clinical lesions in a patient who is not receiving treatment. Prior informed consent was obtained from all patients. Punch biopsies, 3 mm in diameter, were taken from multiple sites: the volar

Correspondence: P.M.Collier, Department of Dermatology, Bristol Royal Infirmary, Bristol BS2 8HW, U.K.

Table 1. Patient demography

Patient	Sex	Age at onset	Disease activity	Therapy*
1	F	49	Active	Dapsone
2	F	69	Active	Dapsone plus SMP
3	F	44	Remission	Nil
4	F	45	Active	Dapsone
5	M	88	Active	Dapsone
6	F	75	Active	Dapsone
7	F	24	Active	Dapsone
8	M	56	Remission	Nil
9	F	1·5	Active	Dapsone plus SMP
10	F	72	Active	Dapsone
11	M	52	Remission	Nil
12	F	60	Active	Dapsone
13	F	67	Active	SMP
14	M	83	Active	Dapsone
15	M	59	Active	Dapsone
16	M	57	Remission	Nil
17	F	18	Active	Dapsone

*Treatment at time of biopsy.
Remission, no clinical lesions and off all treatment; SMP, sulphamethoxypyridazine

Table 2. Direct immunofluorescence at different anatomical sites

Patient	Lip	Forearm	Thigh	Buttock
1	+	+	+	+
2	+	+	+	+
3	−	−	−	−
4	+	−	+	+
5	ND	+	+	+
6	ND	+	+	+
7	+	−	ND	ND
8	−	−	ND	ND
9	+	+	+	+
10	ND	+	+	+
11	ND	+	+	+ (weak)
12	ND	+	+	+
13	ND	+	+	+
14	+	+	+	+
15	+	+	+	+
16	−	−	−	−
17	+	+	+	+

+, positive at this site; −, negative; ND, not done.

surface of the forearm, the outer aspect of the thigh, the buttock, and the mucosal surface of the lower lip. In some patients it was not possible to biopsy all sites simultaneously. The biopsies were snap-frozen, and direct immunofluorescence performed using standard techniques.

Results

The results are shown in Table 2. The majority of patients showed consistent results at all sites. Four patients were in remission at the time of the study, and biopsies from three of these patients (3, 8 and 16) were negative. However, in two patients (4 and 7) the results showed variation, with negative forearm biopsies, but positive biopsies at other sites. Both these patients had active disease and were on treatment at the time of the study. We are unaware of any factors which could account for this finding.

Discussion

The majority of patients did not show any variation in antibody deposition, whether present or absent, between sites, including the oral mucosa. The patients with active disease (either with lesions present, or relapsing on reduction of therapy) in this study showed positive immunofluorescence at all sites tested, including the mucosa, with the exception of two patients who were negative at the forearm site. These findings contrast with studies in pemphigus where no variation has been found.[5] However, there is some evidence to suggest regional variation in bullous pemphigoid.[7–9] The common sites for clinical disease in LAD are the trunk, limbs and mucosae,[11] and as the arm is frequently affected, it is surprising that negative results were obtained at this site. A similar dichotomy has been observed in the conjunctiva, where negative immunofluorescence is found, despite frequent clinical involvement.[4]

In three patients who were in remission, immunofluorescence was negative at all sites, but in a fourth patient, also in remission, the immunofluorescence remained positive. An interesting feature of this disease is the observation that many patients go into remission. Whether all patients in remission eventually lose their immunoreactants is not known, but in a previous study the majority (71%) lost their immunoreactants.[11] Negative immunofluorescence is not a requirement for remission to occur.

These findings suggest that, if a single diagnostic biopsy is to be taken, it would seem prudent to avoid using the volar surface of the forearm (a site often favoured by dermatologists) until further information is available.

Acknowledgment

This work was supported by a grant from the Oxfordshire Health Authority District Research Committee.

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British Journal of Dermatology (1992) 127, 485–491.

Prenatal diagnosis in a subset of trichothiodystrophy patients defective in DNA repair

A.SARASIN, C.BLANCHET-BARDON,* G.RENAULT, A.LEHMANN,† C.ARLETT† AND Y.DUMEZ‡

Laboratory of Molecular Genetics, Institut de Recherches Scientifiques sur le Cancer, B.P. N° 8, 94801 Villejuif Cedex, France
**Cliniques des maladies cutanées (Prof. L Dubertret), Hôpital Saint-Louis, 75010 Paris, France*
†MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton BN1 9RR, U.K.
‡Maternité Port-Royal, Hôpital de Port-Royal, 75005 Paris, France

Accepted for publication 12 June 1992

Summary Trichothiodystrophy (TTD) is an autosomal recessive disorder characterized by brittle hair with reduced sulphur content, and mental and physical retardation. Numerous additional clinical features may be present, producing a very heterogeneous syndrome. Many cases exhibit ichthyosis and photosensitivity. Cells from photosensitive TTD patients show reduced DNA repair levels similar to those found in xeroderma pigmentosum. TTD patients have a short life expectancy, and no treatment is known or envisaged. We report the prenatal diagnosis of TTD in two French families, based on DNA repair measurements in trophoblasts or amniotic cells, with later confirmation by microscopic analysis of the fetal hairs. Although the DNA repair defect was less marked in the fetal cells when compared with fibroblasts from the index case, measurement of DNA repair by unscheduled DNA synthesis provided unambiguous evidence of defective DNA repair in the fetal cells. This method is therefore a suitable prenatal diagnostic test for those TTD families in which a DNA repair defect has been identified.

Trichothiodystrophy (TTD) is an autosomal recessive disease characterized by brittle hair with reduced sulphur content.[1] Clinically, the hair is dry and sparse, and the hair shafts break easily.[2] TTD patients usually show a 10–50% decrease in cystine/cysteine in hair proteins, compared with normal. Affected children often have an unusual facial appearance, and physical and mental retardation.[3] Classification of TTD patients appears very difficult, probably because of the large variety of clinical features in addition to the common hair abnormalities. Thus, some patients have severe ichthyosis, especially at birth, and about 50% show photophobia and a marked sensitivity to sunlight. However, development of skin cancer has not yet been reported. This latter characteristic is important, because it has recently been shown that some TTD patients have a defect in DNA repair very similar to that found in the highly cancer-prone disorder xeroderma pigmentosum (XP).[4,5] Recently, Lehmann and co-workers, analysing cellular responses to UV, found a remarkable heterogeneity amongst fibroblasts isolated from different TTD patients. Three separate classes of TTD cells were demonstrated when various cellular responses to UV were compared.[6,7] Cells of type 1 had a normal UV-response, while those of type 2 were very deficient in DNA repair, with genetic and biochemical characteristics indistinguishable from XP complementation group D. The DNA repair properties of cells of type 3 were intermediate between types 1 and 2. However, cell strains in this class had a reduced rate of repair of pyrimidine (6-4) pyrimidone photolesions.[7] Approximately 70% (17 out of 24) of cell strains, examined either in our own laboratories[6,7] (Broughton *et al.*, unpublished data) or by Stefanini and colleagues,[5,8,9] had defective DNA repair. In general, there was a correlation between photosensitivity in the patients and defective DNA repair.

Because TTD patients die at an early age, and no treatment has been proposed, prenatal diagnosis is important. Prenatal diagnosis of XP has been achieved by the use of a DNA repair assay,[10,11] which should also be applicable to those families in which TTD is associated with defective DNA repair. We report the first prenatal diagnosis in two French TTD families, in which cells from index cases were severely deficient in UV-induced DNA repair, and very sensitive to the lethal action of UV-light

Correspondence: Dr A.Sarasin, Laboratory of Molecular Genetics, Institut de Recherches Scientifiques sur le Cancer, B.P. N° 8, 94801 Villejuif Cedex, France.

and UV-induced inhibition of RNA synthesis. In one case, the diagnosis was confirmed by showing the typical bright and dark bands on fetal hair analysed by polarizing microscopy.

Case reports

Case 1 (Family 1)

The patient was a 21-year-old woman in her second pregnancy. Her first child, a girl, now aged 4 years (index case 1), was born at 39 weeks' gestation with a markedly reduced weight, cranial circumference, and body length. At birth she had the typical features of a collodion baby, and her hair was short and brittle. At the age of 4 years (Fig. 1a), her weight and height were below the third percentile, she was mentally retarded and her skin was severely ichthyotic. Hair examination under the polarizing microscope revealed alternating bright and dark areas (Fig. 2a). The parents sought prenatal studies because they felt that their first child's disease was not compatible with a normal life. They decided to proceed

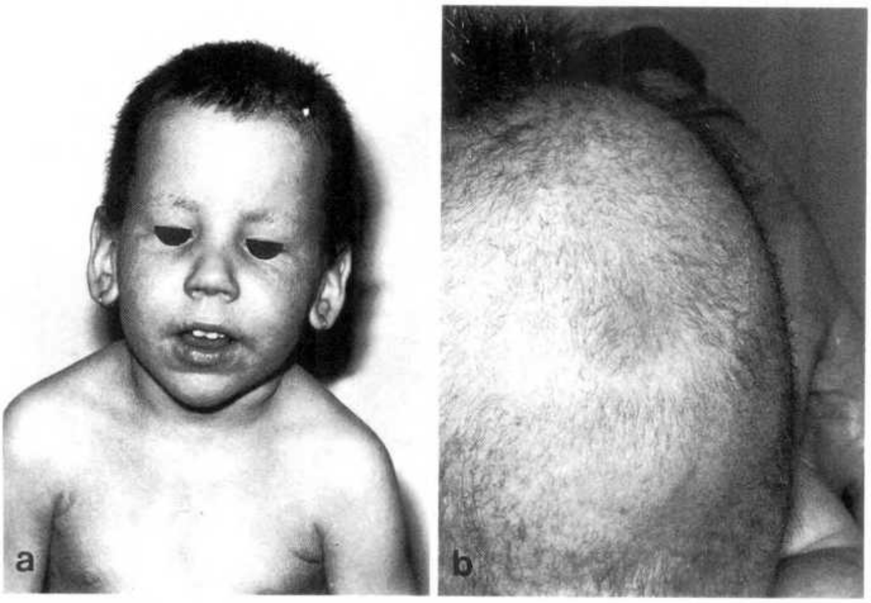

Figure 1. (a) Index case 1 at 4 years of age showing typical face and short brittle hair. (b) Index case 2 at the age of 9 months showing the typical brittle hair.

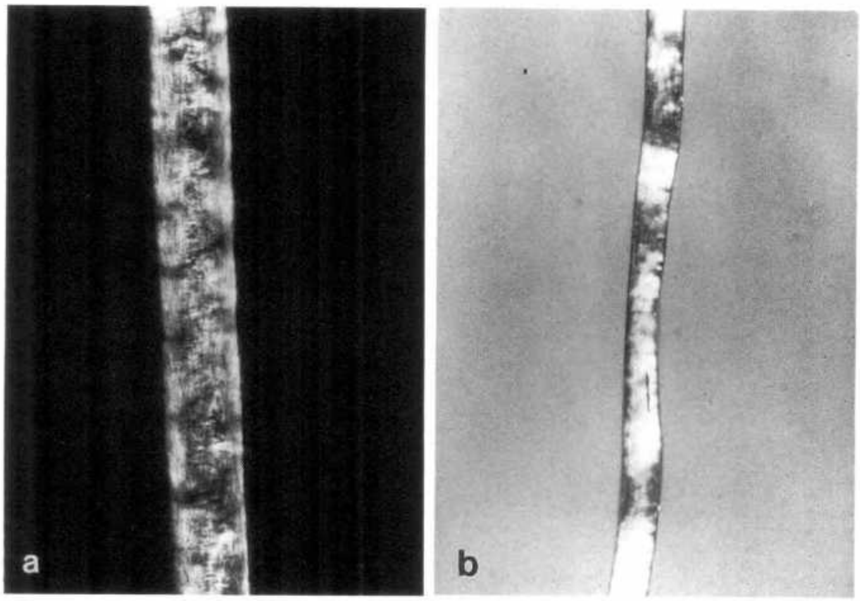

Figure 2. Analysis of hair from index case 1 (a) and of fetal hair from Family 1 (b) by polarizing microscopy. Examination of the hair shaft from the fetus aborted at 26 weeks shows alternating bands of bright and dark areas (b) similar to the TTD child (a).

with the second pregnancy only if prenatal testing revealed no evidence of trichothiodystrophy.

As a result of the studies reported below, the fetus was aborted after 26 weeks' gestation. Fetal skin was examined with light and electron microscopy. Hair was examined by polarizing microscopy, and biochemical analysis of nails and hair was performed for amino acid composition. Similar microscopic and biochemical studies were performed on normal age-matched controls.

Case 2 (Family 2)

The patient was a 30-year-old woman in her third pregnancy. Her first child, a boy, was born at 36 weeks' gestation with markedly reduced weight (1550 g), cranial circumference and body length. His skin was dry and scaly at birth, and his hair was short and brittle. At the age of 4 months he had spastic neurological abnormalities, a congenital cataract, and profound growth retardation. At the age of 6 months, the parents noted severe photosensitivity. This child was never able to sit up or walk. He died at 22 months from cachexia and dehydration. Her second child was born at 33 weeks' gestation with markedly reduced weight (1480 g), cranial circumference and body length. At birth he had scaling feet, and his hair was short and brittle. This child (index case 2) was first seen by us at the age of 9 months (Fig. 1b). His weight and height were below the third percentile, and he was unable to sit up. He had bilateral cataracts. His skin was moderately ichthyotic, and there were nail abnormalities. The parents had noted severe photosensitivity. Hair examination under the polarizing microscope revealed alternating bright and dark areas. There was a deficiency of sulphur-containing amino acids; the cystine content of the hair shaft was reduced by about 30%. He also had spastic neurological abnormalities and he died at the age of 12 months from cachexia and septicaemia.

Methods

Cell cultures

Cell cultures from trophoblast (chorionic villi), fetal amniotic cells or adult skin biopsies were established using Ham's F10 medium supplemented with 20% fetal calf serum (specifically selected for that purpose) and antibiotics. Several different morphological types of cells are clearly visible in fetal cultures, but we found no differences in their relative UDS efficiencies. The origins of the cell lines used are described in Table 1.

Cell survival after UV-irradiation

Cell survival was measured by the colony-forming ability of cells following UV-irradiation, as described by Lehmann *et al.*[12]

Table 1. Origin of cell strains used

		Patients	
Cell strains	Clinical description (phenotype)	Age (years)	Sex
Family 1			
TTD HM2VI (fibroblast)	TTD mother (normal)	21	F
TTD HF2VI (fibroblast)	TTD father (normal)	30	M
TTD 2VI (fibroblast)	TTD	4	F
Am 2VI (amniocyte)	TTD*	Fetus (21 weeks)	
Family 2			
TTD HM1VI (fibroblast)	TTD mother (normal)	30	F
TTD HF1VI (fibroblast)	TTD father (normal)	30	M
TTD 1VI (fibroblast)	TTD	9 months	M
Tr 1VI (trophoblastic cell)	TTD*	Fetus (9 weeks)	
Controls			
TR-Norm (trophoblastic cells)	Normal	Fetus (9 weeks)	
Am-Norm (amniocyte)	Normal	Fetus (21 weeks)	
Norm (fibroblast)	Normal	4	M

* Based on UDS results in this paper.

DNA repair measurements by unscheduled DNA synthesis

Low serum, coverslip cultures of skin fibroblasts, trophoblast cells or amniocytes were prelabelled with 370 KBq ^{3}H thymidine (284 GBq/mmol, NEN) per ml 1 h before UV-irradiation. The coverslip cultures were then washed twice with PBS and irradiated under a germicidal lamp (mainly at 254 nm) with a dose rate of 0·12 J/m^2/s, at the doses indicated. The cells were further labelled in growth medium containing ^{3}H thymidine for 2 h, followed by a 1-h chase incubation with cold thymidine (10^{-5} M). The coverslip cultures were then washed, fixed in methanol and dried. The mounted coverslip cultures were treated with Ilford Nuclear Research emulsion and exposed for 1 week at 4°C. After development, the cells were stained with Giemsa and the average number of grains over the nucleus scored, cells undergoing replication synthesis being excluded.

RNA synthesis measurements

The procedure used was that of Mayne and Lehmann.[13] Briefly, low serum cultures in 3·5-cm Petri dishes were UV-irradiated. The following day ^{3}H uridine was added to the culture medium for 4 h. SDS cell lysates were deposited on Whatman 3MM paper, washed in TCA, rinsed in ethanol, dried and counted for radioactivity. Results are given as the ratio of radioactivity incorporated in irradiated cells to that in unirradiated cells.

Results

Cellular studies

Index cases. We have tested the cellular responses to UV-light in the two families. Skin fibroblasts from the two TTD index cases (TTD 1VI and TTD 2VI) are much more sensitive to UV-light compared with the heterozygote parent cells or normal individuals (Fig. 3). Similarly, the efficiency of UV-induced DNA repair has been studied by measuring UDS in skin fibroblasts isolated from parents and index cases. Figure 4(a) and (b) show that both index cases (TTD 1VI and TTD 2VI) had very low levels of UV-repair (about 20% of normal) compared with the parental heterozygotes, which were in the normal range. Both cell strains are therefore assigned to type 2.[7] The easily detected differences between parent heterozygote and TTD-homozygote cells provide a possible procedure for prenatal diagnosis.

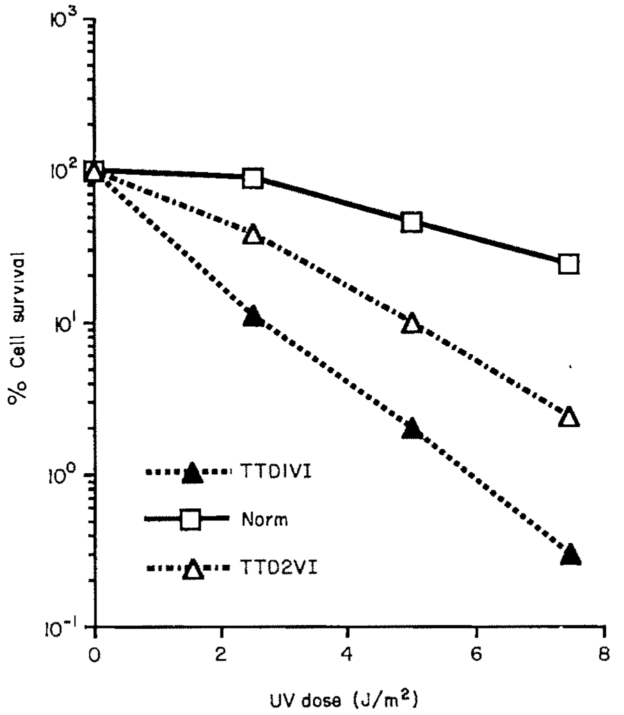

Figure 3. UV-survival of TTD strains. Growing cells were UV-irradiated and then plated at low density to measure their colony-forming ability. Cell lines are described in Table 1.

Case 1: amniocytes. In Case 1 cells from amniotic fluid were taken at 21 weeks of pregnancy and established in culture (Am 2V1). UV-irradiation of this cell line produced a low level of DNA repair compared with control amniotic cells (Am-Norm) established from a normal pregnant woman (Fig. 5). Termination of pregnancy was requested very late by the parents, and was performed with prostaglandins. At delivery the fetal skin was covered with a collodion membrane. Hairs were sparse, brittle and extremely short. Hair examination with a polarizing microscope revealed alternating bands of bright and dark areas, typical of TTD hairs (Fig. 2b).[2] This confirmed that the fetus was suffering from the TTD syndrome, because the hair abnormalities were characteristic (Fig. 2a). These results indicated that prenatal diagnosis of TTD was possible, although for non-medical reasons investigations were performed very late in pregnancy. Therefore, we decided to determine if such a test was possible earlier, for example on a trophoblast biopsy performed at 8–10 weeks' gestation.

Case 2: trophoblasts. In Case 2 a trophoblast biopsy was performed at 9 weeks, fetal cells were cultured for 4

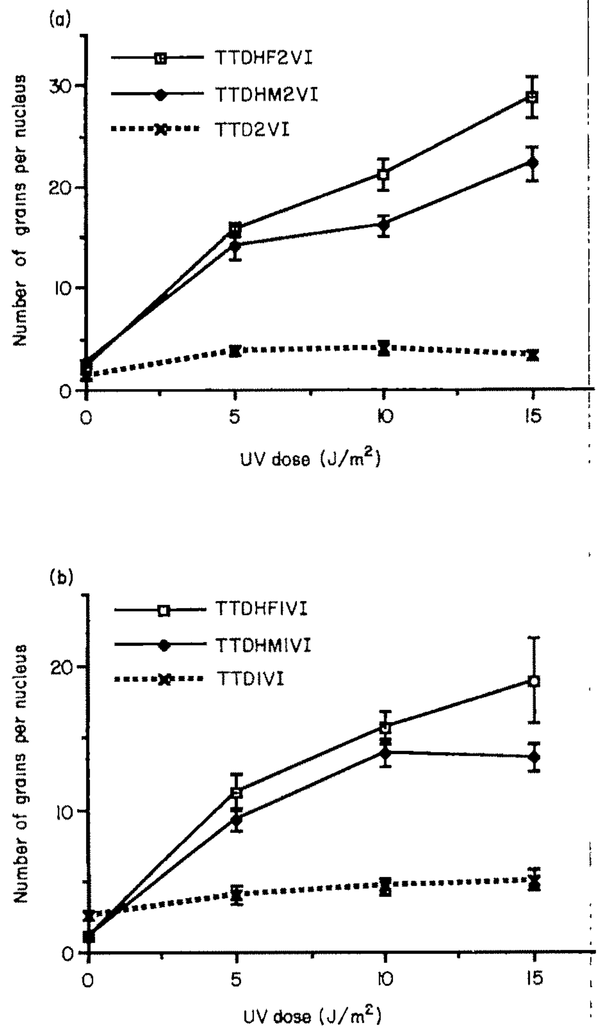

Figure 4. Levels of UDS induced after increasing doses of UV in TTD families. (a) Family 1, (b) Family 2. Cell nomenclature is according to Table 1. UDS was performed as described in Methods, and quantified as number of grains per nucleus as a function of UV dose. Error bars correspond to ±SE.

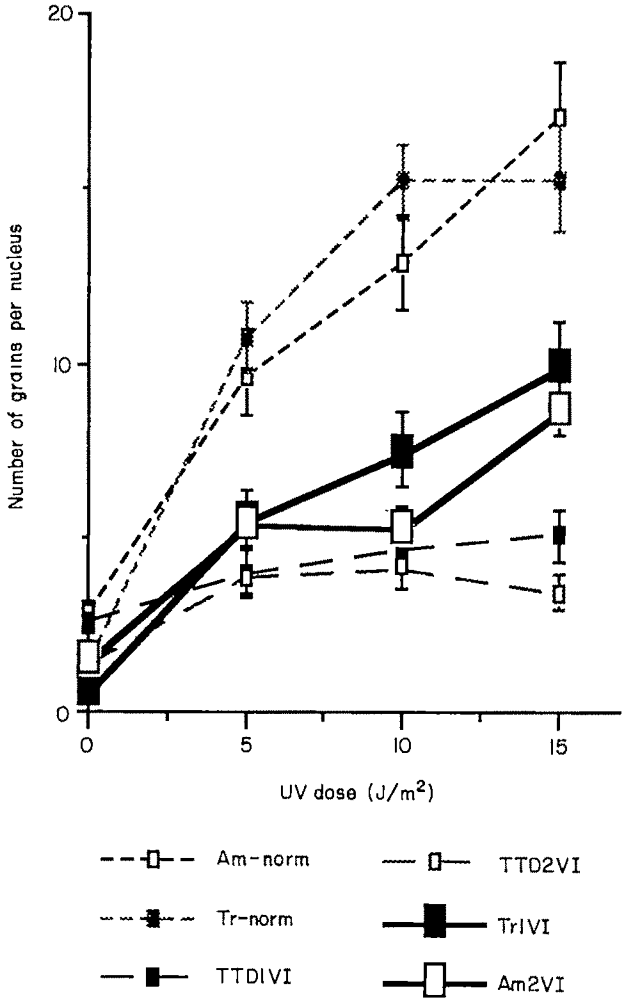

Figure 5. Levels of UDS induced by increasing doses of UV in fetal cells and in fibroblasts of index cases. Cell nomenclature is according to Table 1. UDS was performed as described in Methods, and quantified as number of grains per nucleus as a function of UV dose. Error bars correspond to ±SE.

weeks, and UDS analysis was carried out. Figure 5 shows that trophoblastic cells (Tr 1VI) exhibited a low DNA repair level after UV-irradiation, compared with trophoblastic cells from a pregnancy tested for other reasons (Tr-Norm). This result was confirmed by several independent measurements of UV-induced repair using UDS. Unfortunately, due to a spontaneous abortion, no fibroblastic cell line could be derived from the fetus.

We also examined the level of RNA synthesis inhibition after UV-irradiation of the trophoblast culture, because this parameter has been shown to be a useful and sensitive indication of UV sensitivity in several DNA repair syndromes, including repair-deficient TTD.[7] As expected, the index case exhibited a marked decrease of transcription after UV-irradiation, but, surprisingly, the transcriptional activity in the trophoblast culture, although reproducibly slightly lower than in heterozygote lines at high doses, was not significantly different from control trophoblasts (Fig. 6).

Discussion

Our results show that prenatal diagnosis of TTD can be performed in families at risk by measuring the level of

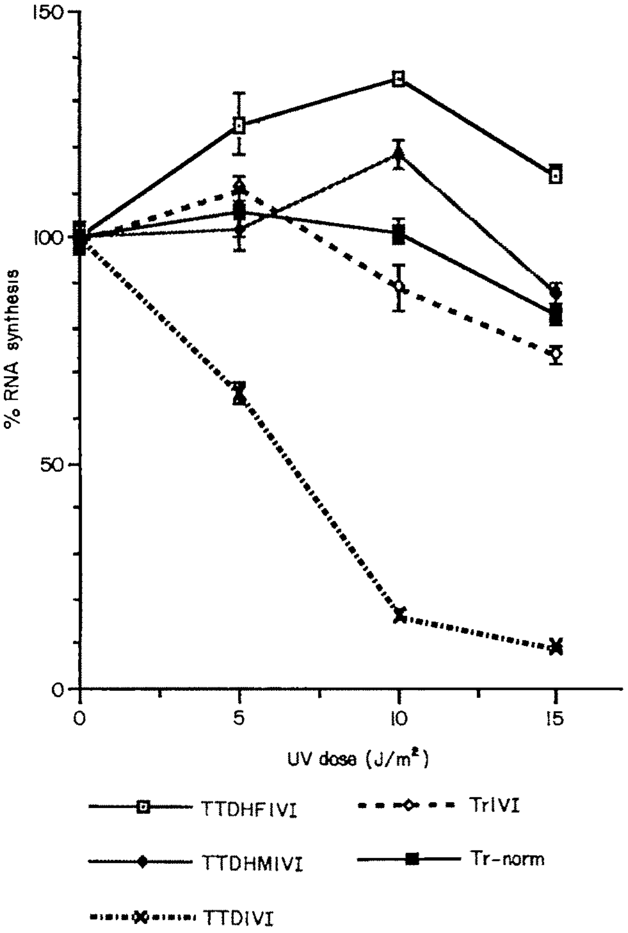

Figure 6. Inhibition of RNA synthesis by increasing doses of UV in fetal and adult cells in TTD Family 2. RNA transcription was quantified according to Lehmann *et al.*,[6] and is expressed as % of unirradiated values.

UDS after UV-irradiation. This procedure is limited to those TTD families in which the disorder is known to be associated with defective DNA repair. Because such an association is not found in all families (an estimate from our limited information is that about 70% of cases are repair-deficient), it is absolutely essential to determine the cellular responses to UV of the cells of the index case compared with controls, and with the parental heterozygote cells prior to carrying out prenatal diagnosis. Only if a clear defect is identified in the index case can prenatal diagnosis be proposed.

It is interesting to note (Fig. 5) that the UV-induced UDS level of fetal cells appears in both cases to be higher than that observed with the index case fibroblasts. Moreover, it is also obvious from Figure 6 that the RNA transcription is much less UV-sensitive in trophoblastic cells than in fibroblasts from the index case. These results could be due to relatively trivial reasons concerned with different growth behaviour of fetal cells compared with mature fibroblasts. However, it is possible that these differences may correspond to a decrease in cellular ability to repair UV-induced lesions after birth in the case of repair-deficient TTD. Direct measurements of the rate of removal of lesions would be necessary to resolve this point.

In conclusion, prenatal diagnosis can be performed by following UV-induced UDS on trophoblast biopsy cells in those TTD families in which there is an associated defect in DNA repair. In these cases the result can be provided at about 14 weeks' gestation (approximately 5 weeks after biopsy). This early detection allows possible confirmation of the result either by measurement of UDS in cells from amniotic fluid (16–18 weeks' gestation) or by fetal hair biopsy. The analysis of fetal hairs, which can be carried out at about 20 weeks' gestation, permits absolute confirmation of the diagnosis, because the hair structure abnormality is characteristic of TTD.

Acknowledgment

The authors are grateful to Mrs A.Benoit and A.Margot for technical assistance. This work was supported by the 'Caisse Nationale d'Assurance Maladie des Travailleurs Salariés (Paris), the 'Association Française contre les Myopathies' (Paris) and the 'Association pour la Recherche sur Cancer' (Villejuif, France).

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British Journal of Dermatology (1992) 127, 492–496.

Information leaflets in the dermatology out-patient waiting area

C.C.HARLAND, R.J.MADELEY* AND L.G.MILLARD
*Departments of Dermatology and *Public Health Medicine and Epidemiology, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH, U.K.*

Accepted for publication 1 June 1992

Summary

Patients' attitudes to the supply and quality of information provided about their condition were examined by questionnaire before ($n=852$) and after exposure to an information leaflet campaign ($n=560$) in the dermatology out-patient waiting area. A high expectation of this service was demonstrated, particularly from patients under 60 years of age with chronic diseases. A variety of information sources, other than doctors, were identified which could be improved (video-systems, specialist nurses, leaflets, posters). The campaign significantly increased the percentage of patients who derived information from the leaflet source (5·6–18·8%, $P<0{\cdot}001$) and it reduced a demand for leaflets (16·4–9·1%, $P<0{\cdot}001$). However, the campaign did not alter patients' perceived need to spend more time with a dermatologist. In order to achieve a greater impact on patient satisfaction, a combination of information sources in the out-patient department should be targeted at young adults with chronic diseases.

According to their patients, doctors fail to provide adequate information.[1,2] However, the medical profession is increasingly recognizing that improved communication leads to better treatment compliance and patient satisfaction.[2,3] Consequently, there has been a recent upsurge of interest in printed information for patient education.[1,4–7]

Despite the availability of patient information leaflets in most dermatology out-patient departments, there is little reported data supporting their use. Printed information for dermatology out-patients receiving PUVA therapy has been shown to contribute to greater knowledge of their particular skin disease and of PUVA therapy.[8] Patients with contact allergy have also benefited from a detailed information list about specific allergens and their avoidance; clearance of eczema was found to correlate with the use of the information list.[9] However, the role of leaflets and other information sources in the out-patient department has not been adequately evaluated.

This study examined patients' attitudes to various sources of information about their condition. The impact of an information leaflet campaign on patients' satisfaction was assessed.

Patients and methods

Follow-up patients in the adult dermatology out-patient department at Nottingham were asked to complete a questionnaire before and after exposure to an information leaflet campaign. Questionnaires were issued by the clinic receptionist to two groups of patients as they arrived for their follow-up appointment. The first group consisted of those patients who had attended the clinic during the 4-week period preceding the campaign, when the waiting area was devoid of dermatological literature. The second group comprised those who had been exposed to the campaign during a previous visit. Although patients received only one questionnaire during each study period, overlap of patients between each group occurred where clinic visits were frequent.

The campaign involved a constant supply of leaflets, which were displayed in the waiting area for 4 months. The leaflets covered over 20 topics relevant to out-patient dermatology care (Table 1), and their design fulfilled recommended guidelines for clarity of communication.[1,6,7] Many of these leaflets were purchased in bulk from societies and associations. To encourage their perusal, leaflets were arranged on display modules in the waiting area. Irrespective of their involvement in the questionnaire study, all patients, relatives and visitors were allowed to remove leaflets from the department.

Sources of information were 'family doctor', 'hospital doctor', 'clinic nurse', 'other patients', 'leaflets', 'posters', 'societies and associations', and 'others'. Patients rated these from 'very poor' to 'excellent', and indicated whether, and through which information source, the information needed to be improved. They were also

Correspondence: Dr C.C.Harland, Department of Dermatology, St Helier Hospital, Wrythe Lane, Carshalton SM5 1AA, U.K.

Table 1. Diagnostic categories and topics covered by study leaflets

Diagnosis	Title	Leaflet source
General	*Common skin problems*	British Medical Association
Acne vulgaris	*The facts about acne*	Schering Health Care Ltd*
	Your acne. Your questions answered	Quinoderm Ltd*
	All about acne	Pfizer Ltd*
	Learning to live with acne	Roche Products Ltd*
	Answers to questions about acne care	Upjohn Ltd*
	Understanding acne	Good Health magazine*
Psoriasis	*Learning to live with psoriasis*	Roche Products Ltd*
	Psoriasis. Your questions answered	Quinoderm Ltd*
	Beyond the ointment	The Psoriasis Association*
	What is psoriasis?	The Psoriasis Association*
Eczema	*Eczema*	Department of Dermatology, Nottingham
	Exchange	National Eczema Society*
	Questions need answers	National Eczema Society*
	Looking after your hands	Neutrogena (UK) Ltd*
Nappy rash	*Everything you need to know*	Bayer (U.K.) Ltd*
Leg ulcers	*Doctor, it's my leg again*	Hoechst Ltd*
	Leg ulcers	Squibb Surgicare Ltd*
	Working together on the road to recovery	Perstorp Pharma Ltd*
	Legs eleven	Department of Dermatology, Nottingham
Moles	*Be a mole watcher*	Cancer Research Campaign*
Skin cancer	*It's never too early to stop skin cancer*	The Skin Cancer Foundation, U.S.A.
	Types and descriptions of skin cancers	The Skin Cancer Foundation, U.S.A.
Basal cell carcinoma	*Basal cell carcinoma*	The Skin Cancer Foundation, U.S.A.
Malignant melanoma	*Guidelines and early warning system*	The Skin Cancer Foundation, U.S.A.
Dysplastic naevi	*Dysplastic naevi and melanoma*	The Skin Cancer Foundation, U.S.A.
Sun exposure	*Simple guidelines to help protect you*	The Skin Cancer Foundation, U.S.A.
	For every child under the sun	The Skin Cancer Foundation, U.S.A.
Warts	*All about warts*	Dermal Laboratories Ltd*
Verrucae	*Verrucae*	The Society of Chiropodists
Hair disorders	*Hair growth and hair loss*	Upjohn Ltd*
Urticaria	*Urticaria*	Department of Dermatology, Nottingham
Pruritus	*Itching*	Department of Dermatology, Nottingham
Scabies	*What everyone should know about scabies*	Stafford-Miller Ltd*
Allergies	*Seasonal allergies*	Kirby-Warwick Ltd*
Vitiligo	*Vitiligo*	Vitiligo group*
Topical steroids	*Steroid creams*	Department of Dermatology, Nottingham
Emollients	*You and your skin*	Dermal Laboratories Ltd*
PUVA	*PUVA treatment*	Stiefel Laboratories Ltd*

*Gifts to the Department of Dermatology, Nottingham, U.K.

asked if a video-programme about their particular condition should be available in the waiting area.

The responders were divided into eight diagnostic categories ('acne', 'psoriasis', 'eczema', 'tumours', 'leg ulcers', 'warts', 'other', and 'don't know'). The tumour group included those patients who considered that their presenting complaint had been a mole, skin growth or cancer.

Statistical methods

An SPSS computer program was used for statistical analysis of questionnaires. Responses were compared by χ^2 test with Yates' correction, and by the test for two proportions where the responses for different diagnostic categories were compared.

Results

There were 852 respondents before, and 560 after, leaflet exposure (95% uptake of questionnaires). The two groups did not differ significantly in age, sex, social class or diagnosis. Table 2 shows a percentage breakdown of patients' diagnoses. Some patients (17%) did not know their diagnosis, and this was unaffected by the campaign. A large number of leaflets (6630) were taken away from the department (approximately £300 was spent on the replenishment of leaflets).

Overall information was rated as 'good' or 'excellent' by 72·3% of patients (Table 3). The majority received their information from the hospital or family doctor. Despite this, 47·2% of respondents indicated that the overall information should be improved (Table 4), and 36·3% wanted more time discussing their skin problem with the hospital doctor (Table 5). Also, 18·2% wanted to watch a video about their complaint. The campaign did not alter these views significantly. Only 5·6% obtained information from leaflets (Table 3), but this increased to 18·8% following the campaign ($P<0{\cdot}001$). 16·4% of patients wanted better leaflet information before, and 9·1% after, the campaign ($P<0{\cdot}001$) (Table 5). Table 4 shows that patients who were under 60 years of age were less satisfied with the overall information compared with those over 60 ($P<0{\cdot}001$). The responses for chronic disorders (eczema, psoriasis and acne) were compared with those from the tumour group: 125 of 208 (60·1%), 156 of 233 (67·0%) and 55 of 95 (57·9%), respectively, had a greater perceived need for information, as opposed to 64 of 172 (37·2%) in the tumour group ($P<0{\cdot}001$).

Table 2. Diagnostic categories

Diagnostic category	Responders (%)*
Psoriasis	18
Eczema	16
Tumours	14
Warts	8
Acne	7
Leg ulcers	5
Other	19
Don't know	17

*Patients allowed to indicate more than one diagnosis.

Table 3. Analysis of follow-up patients' information source, and satisfaction with information provided about their diagnoses, before and after exposure to leaflet campaign (no. (%) of patients)

	Before ($n=852$)	After ($n=560$)
Information source		
Family doctor	482 (56·6)	317 (56·6)
Hospital doctor	616 (72·3)	395 (70·5)
Leaflets	48 (5·6)	105 (18·8)*
Clinic nurse	53 (6·2)	36 (6·4)
Patients	57 (6·7)	44 (7·9)
Posters	28 (3·3)	32 (5·7)
Societies/associations	18 (2·1)	20 (3·6)
Other	71 (8·3)	43 (7·7)
Overall information		
Excellent	247 (29·0)	126 (22·5)
Good	364 (42·7)	249 (44·5)
Average	154 (18·1)	119 (21·2)
Poor	38 (4·5)	26 (4·6)
Very poor	10 (1·2)	3 (0·5)

*$P<0{\cdot}001$.

Table 4. Patients' attitudes to the overall information about their skin complaint, before and after exposure to leaflet campaign, according to age (no. (%) of patients)

	Improvement of overall information needed	
Age category	Before ($n=852$)	After ($n=560$)
All ages	402 (47·2)	263 (47·0)
<60	303 (35·6)	205 (36·6)
$\geqslant 60$*	99 (11·6)	58 (10·4)

*$P<0{\cdot}001$.

Table 5. Information sources which should be improved according to out-patients, before and after exposure to leaflet campaign (no. (%) of patients)

	Improvement of overall information needed	
Information source	Before ($n=852$)	After ($n=560$)
Family doctor	146 (17·1)	85 (15·2)
Hospital doctor	309 (36·3)	193 (34·5)
Leaflets	140 (16·4)	51 (9·1)*
Clinic nurse	54 (6·3)	34 (6·1)
Posters	48 (5·6)	29 (5·2)
Societies/associations	58 (6·8)	40 (7·1)
Videos	155 (18·2)	108 (19·3)

*$P<0{\cdot}001$.

Discussion

This study demonstrates a high expectation for information amongst dermatology out-patients in Nottingham. The overall information supplied before and during the leaflet campaign was considered to be good or excellent by more than two-thirds of patients, yet nearly half of respondents wanted an even better provision of information. Younger patients (<60 years) were more demanding of this service. These findings reflect society's growing awareness of health and education,[4,10,11] as well as the growth of consumerism in the National Health Service.[12] It is likely that attitudes of the under-60 population are influenced more than those of older patients.

In addition, the skin condition itself affected patients' opinions about their education. Patients with chronic skin diseases (eczema, psoriasis, acne) were more demanding of information than the tumour group. This difference was still significant when allowances were made for age, the latter group being older. The tumour group generally required reassurance about moles or cosmetic procedures for benign tumours, or other curable conditions. On the other hand, patients with acne, eczema and psoriasis appear to have been more demanding because of the chronic, relapsing nature of their disease. Future education campaigns should arguably be directed towards such conditions.[13] Some caution is necessary in interpreting these findings as, paradoxically, those who complain about lack of information may be the best informed.[14]

The majority of patients received information from doctors. Individually, other information sources are relatively unimportant, but, cumulatively, they may be valuable for patient education. The implementation of a leaflet campaign resulted in an improved service. Although the perceived need to spend more time with the dermatologist was not influenced by the campaign, significantly more patients derived information about their complaint when leaflets were readily available in the waiting area, and the campaign also fulfilled a demand for leaflets. Table 2 shows that the percentage of patients who used leaflets rose from 5·6 to 18·8 ($P<0{\cdot}001$) as a result of the campaign. Table 3 demonstrates a reduction in the percentage of patients who wanted an improvement of leaflet advice following the campaign ($P<0{\cdot}001$).

Other information sources (clinic nurse, posters, videos) could be targeted concurrently at patients. Education by specialist nurses in a dermatology department or within the community may be an important means of improving this service. Interestingly, 18% of respondents wanted video-information. Video- and audiotape information packages about specific diseases or treatments have not been adequately evaluated in the out-patient department, but there are indications that these are effective for presenting information, and may reduce consultation times.[15] A video-replay system in the waiting area enables prerecorded health-education tapes to be played to patients, and has been assessed in a general practice waiting area,[16] where 58% of attenders found it useful. Other public services have shown the value of the television screen as an information source, including travel (railways and airports), shopping (video demonstrations), and leisure (museums, etc.).

Video-systems may be superior to the provision of leaflets in terms of cost-benefit ratio. Over 6000 leaflets were replenished during the 4-month campaign at a cost of about £300. As leaflets were available to all patients (new and follow-up), relatives and visitors, the number removed by the study group was probably much less. Also, only a small proportion of follow-up patients benefited from leaflet exposure. For example, the percentage of patients who wanted an improvement in leaflet information was reduced from 16·4 to 9·1%, so that 7·3% were apparently satisfied by the provision of leaflets. Therefore, 41 of 560 responders might have benefited at a cost of around £7 per person. Cost-benefit could be improved by photocopying standardized printed sheets, subject to copyright. Posters, stated as a source of information by 3·3–5·7% of patients (Table 3), could be a relatively inexpensive adjunct to leaflets.

Leaflets and other audiovisual aids merit further attention as a means of improving the out-patient service. Because doctors are viewed by patients as the primary source for information, leaflets may be best delivered by the doctors themselves in the consultation room to achieve most impact. However, leaflets and other information sources should be regarded as a supplement, not a substitute, for good communication between the doctor and the patient. It is worrying that 17% of follow-up patients in this study did not know their diagnosis. This deficiency of communication should be detected and rectified by the doctor.

Acknowledgments

We are grateful for advice and support from Ian D.Turner, Department of Public Health, Medicine and Epidemiology.

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British Journal of Dermatology (1992) 127, 497–500.

Contact hypersensitivity to topical corticosteroids

A.D.BURDEN AND M.H.BECK
The Skin Hospital, Chapel Street, Salford M60 9EP, U.K.

Accepted for publication 10 June1992

Summary Topical corticosteroids are increasingly recognized as relatively common contact sensitizers. Between July 1988 and December 1991 2687 patients undergoing routine patch testing were also tested with tixocortol pivalate (TP). Over the same time period 528 patients were selected for testing with a series of 18 steroids. One-hundred and thirty-one cases (4·9%) of corticosteroid hypersensitivity were detected and 119 (90·8%) of these cases were positive to TP. Thirty-seven patients reacted to one or more steroids in the steroid series, the most frequent sensitizers being hydrocortisone, budesonide (3·6%) and hydrocortisone 17-butyrate (2·5%). Of these 37 cases, 20 (54%) reacted to more than one steroid simultaneously, but the patterns of cross-reaction were not consistent with previously suggested groupings. Screening for steroid allergy should be performed as part of standard patch testing. The value of TP as a marker of corticosteroid hypersensitivity is reinforced by this study, but no satisfactory marker was found for the 9·2% of cases not detected by TP. There remains a need for further markers of corticosteroid hypersensitivity. A prevalence of 4·9% of corticosteroid allergy amongst our patients suggests that the frequency of this finding is generally underestimated.

Over the 33 years since it was first reported,[1] contact hypersensitivity to corticosteroid molecules has been increasingly recognized as an important cause of allergic contact dermatitis.[2–5] The reported prevalence amongst patients undergoing patch testing varies between 0·2%[5] and 4·8%.[6] Some corticosteroids have been implicated more often than others, and some patients are allergic to more than one corticosteroid. False negative patch-test reactions are troublesome, and have been attributed to the test concentration,[3] choice of vehicle,[4,7] poor absorption[6] and the anti-inflammatory effects of corticosteroids.[7]

Several studies of corticosteroid hypersensitivity have been reported,[2–4,8,9] with variations in the clinical features, steroid molecules and patterns of cross-reaction. Our study, one of the largest so far reported, was undertaken to assess the frequency of corticosteroid allergy by routine patch testing, and to identify the steroids implicated and the patterns of cross-reaction.

Methods

At the Manchester Skin Hospital 2687 unselected patients undergoing routine patch testing for suspected allergic contact dermatitis were also tested with tixocortol pivalate (TP). Between July 1988 and April 1990 TP 0·5–2% in petrolatum was employed, but between April 1990 and December 1991 this was not available and 'Pivalone' nasal spray containing 1% TP was used.

Over the same time period, 528 patients were selected for testing with a steroid series, either on the basis of clinical suspicion of possible steroid allergy, or if a positive reaction to TP developed. The steroid series consisted of most topical corticosteroids commercially available in the United Kingdom. Budesonide and desoxymethasone were only added to the series in October 1990, and 192 patients were subsequently tested. All steroids in the series were tested at 20% in petrolatum (Table 1). Sixty-eight patients were tested on an *ad hoc* basis with a saturated solution of hydrocortisone in alcohol. Tests were performed using Finn chambers on Scanpor tape, and were read at 2 and 4 days. Scoring was in accordance with the ICDRG standards and localized, non-palpable erythematous reactions were not included in the analysis.

Results

Of 2687 patients tested, 131 (4·9%) yielded positive results to one or more corticosteroids. Their age range was 9–87 years (mean 49·1 years), 60 were male and 71 female. The clinical patterns of eczema can be seen in Table 2. Of the 131 corticosteroid-sensitive patients, 119

Correspondence: Dr A.D.Burden, Department of Dermatology, Western Infirmary, Glasgow G11 6NT, U K

Table 1. Positive reactions in patients tested with a series of topical corticosteroids

	TP+ve (119)	TP−ve (12)	Total	%
Hydrocortisone (saturated solution in ethanol)	11	0	11	16·2
Budesonide	6	1	7	3·6
Hydrocortisone 17-butyrate	9	4	13	2·5
Desoxymethasone	0	2	2	1·0
Hydrocortisone (20% petrolatum)	4	1	5	0·9
Clobetasol propionate	1	4	5	0·9
Betamethasone 17-valerate	2	2	4	0·8
Clobetasone butyrate	0	3	3	0·6
Beclomethasone dipropionate	0	2	2	0·4
Flurandrenolone	1	1	2	0·4
Fluocinonide	1	0	1	0·2
Triamcinolone	0	1	1	0·2
Desonide	1	0	1	0·2
Fluocortolone	1	0	1	0·2
Methylprednisolone acetate	0	1	1	0·2
Fluocinolone acetonide	0	0	0	0·0
Diflucortolone valerate	0	0	0	0·0
Halcinonide	0	0	0	0·0
Fluclorolone acetonide	0	0	0	0·0

Table 2. Clinical patterns

Site affected	Number of patients	%
Hand	35	26·7
Foot	12	9·2
Hand and foot	4	3·1
Face	20	15·3
Lower leg (venous)	18	13·7
Perianal and vulval	13	9·9

(90·8%) gave a positive patch-test reaction to TP and 12 were negative. Eighty-eight of the patients who were positive to TP were tested with an extended corticosteroid series (Table 1) and 25 had one or more positive reactions. Each of the 12 patients who were negative to TP reacted to a steroid in the series. Of the 68 patients tested with hydrocortisone in alcohol, 11 (16·2%) were positive, all of whom were also positive to TP. Of the 37 patients who had positive reactions to steroids in the series, 20 (54%) reacted to two or more steroids (Table 3), excluding interactions between TP and hydrocortisone.

When the results of the standard series are analysed in those who are allergic to steroids, it can be seen that 67 (51·2%) had reactions to the constituents of topical medicaments, and these were often multiple (Table 4).

Discussion

Contact hypersensitivity to topical corticosteroids is often not suspected clinically. A clue to its presence is a lack of the expected anti-inflammatory effect of a topical steroid. However, this may also be due to hypersensitivity to other constituents of the preparation, and steroid allergy often occurs in association with allergy to these other constituents, as was found in 51% of the cases in this study. As previously reported,[3,4] neomycin is most frequently involved, but of particular note is cetylstearyl alcohol, a common constituent of steroid creams, which was positive 20 times more frequently in those with steroid hypersensitivity than in the general population undergoing patch testing. The presence of positive reactions to the constituents of topical medicaments, especially multiple positives, may be a pointer to steroid allergy.

It has previously been reported that patients with venous stasis eczema are the commonest clinical group with steroid allergy.[2] Whilst these were a significant group of our patients (13·7%), the majority comprised hand, foot and facial eczema, inevitably reflecting patient selection to some extent. A small but important group were patients with perianal and vulval disease.

The results of this study confirm a high prevalence of contact hypersensitivity to corticosteroids in those undergoing patch testing for suspected allergic contact dermatitis. The figure of 4·9% must represent a mini-

Table 3. Cases of multiple positive patch tests

1	TP (A)*, hydrocortisone 17-butyrate (D)
2	TP (A), hydrocortisone 17-butyrate (D)
3	TP (A), hydrocortisone 17-butyrate (D)
4	TP (A), hydrocortisone 17-butyrate (D)
5	TP (A), hydrocortisone 17-butyrate (D)
6	TP (A), hydrocortisone 17-butyrate (D), fluocinonide (B)
7	TP (A), hydrocortisone 17-butyrate (D), budesonide (B)
8	TP (A), hydrocortisone 17-butyrate (D), budesonide (B)
9	TP (A), hydrocortisone 17-butyrate (D), budesonide (B)
10	TP (A), hydrocortisone 17-butyrate (D), budesonide (B),flurandrenolone (B)
11	TP (A), budesonide (B), fluocortolone (C)
12	TP (A), budesonide (B)
13	TP (A), betamethasone 17-valerate (D)
14	TP (A), betamethasone 17-valerate (D)
15	TP (A), clobetasol propionate (D)
16	TP (A), desonide (B)
17	Hydrocortisone 17-butyrate (D), budesonide (B), desoxymethasone (C)
18	Clobetasol propionate (D), desoxymethasone (C)
19	Clobetasol propionate (D), clobetasone butyrate (D), betamethasone 17-valerate (D), beclomethasone dipropionate (D)
20	Clobetasol propionate (D), clobetasone butyrate (D), hydrocortisone 17-butyrate (D), beclomethasone dipropionate (D), methylprednisolone (A)

* Letter in parentheses refers to steroid groupings suggested by Coopman *et al.*[10]

Table 4. Concomitant positive patch tests to constituents of medicaments in the standard series

	Positive patch test	%	*
Neomycin	41	31·2	(6·0)
Fragrance mix	35	26·7	(7·9)
Caine mix	31	23·6	(2·9)
Quinoline mix	24	18·3	(1·9)
Wool alcohols	24	18·3	(3·0)
Parabens	12	9·2	(1·3)
Cetylstearyl alcohol	9	6·9	(0·3)
Ethylene diamine	7	5·3	(2·1)
Chlorocresol	3	2·3	(0·4)
Bronopol	1	0·8	(1·0)

* Percentage positive in all patients patch tested in the same department.[11]

mum for several reasons: (i) 80% of patients were tested only to TP, which has been shown in this study and elsewhere[12] not to detect all cases; (ii) there may have been false negatives, relating to the concentration and vehicle used, in those tested with the steroid series; (iii) intradermal testing has been shown to detect cases missed by epicutaneous testing;[13] (iv) 'cutaneous paradox' as suggested by Dooms-Goossens *et al.*[7] may operate: the steroid may cause an allergic contact eczema on the affected skin while giving a negative patch test on an intact test site; (v) late reactions may have been missed, as results were read at 2 days and 4 days only. Previous reports have suggested that this should be adequate,[4] but one of our cases, although positive to TP at 4 days, did not react to hydrocortisone 17-butyrate, budesonide, or hydrocortisone in alcohol, until day 12. Alternatively, this patient may have been actively sensitized by the patch-testing procedure.

The frequency with which different steroids are involved varies widely and it has not been established whether this relates to the frequency of use, epidermal penetration, or cross-reaction patterns, or whether some steroids are inherently more allergenic than others. It is well known that many patients react to more than one steroid, and this is again demonstrated in the present study (Table 3). Some multiple positives may represent sensitization to several steroids independently, but true cross-reactions are strongly suggested by positive reactions to steroids which are not commercially available, and by the patterns of multiple positive reactions. However, although patterns of cross-reaction are apparent in this series, they do not generally conform to the four classes suggested by Coopman *et al.*,[10] based on structural variations in the steroid D ring and C20, C21 side-chain. Indeed, other than the interaction between TP and hydrocortisone, in only one patient (Table 3, Case 19) were steroids from the same class involved in all reactions. Of particular note in this study is the interaction, previously noted,[9] between TP and hydrocortisone 17-butyrate, and also between TP, hydrocortisone 17-

butyrate and budesonide. These interactions are important in selecting a steroid with which to treat patients with a known steroid allergy. In patients who are allergic to TP or hydrocortisone it would seem wise to avoid hydrocortisone 17-butyrate and budesonide in particular.

As the prevalence is so high, screening for steroid allergy should be performed as part of a standard patch-testing series. There is a need for markers of steroid allergy, as it is impractical to test all patients to a complete steroid series. Of the various markers which have been proposed, the value of TP is confirmed in this study, identifying 90·3% of all cases detected—63 cases (48%) would have been missed if TP had not been used. It has been suggested that TP is particularly useful as a marker of hydrocortisone hypersensitivity, and of the 13 patients shown to be allergic to hydrocortisone (either in petrolatum or alcohol) in this study, 12 were TP positive. The relevance of positive patch-test results with TP in patients in whom epicutaneous hydrocortisone testing is negative, has previously been established in a study in which 100% of patients who were TP positive were also positive to intradermal hydrocortisone.[6] Although hydrocortisone in alcohol was not assessed systematically in this study, it appears to be inferior to TP, but superior to hydrocortisone in petrolatum, in detecting hydrocortisone allergy. Of interest were two cases in which TP was positive, hydrocortisone in alcohol negative and proprietary hydrocortisone cream in one case and Daktacort® cream in another were positive, other constituents of the cream being negative. It has previously been suggested that the cream base of Daktacort® is a useful vehicle.[7] Budesonide caused a higher rate of positive patch-test reactions (3·6%) than any other steroid, apart from hydrocortisone. Hypersensitivity to budesonide was first reported in 1980 in a patient with psoriasis who developed a dermatitis 2 days after the first application of Preferid® ointment.[14] In 1988 a case was reported from Japan of allergy to budesonide, amongst other steroids, before budesonide was available for therapeutic use in Japan,[15] suggesting a cross-reaction. The only previous report in which a large population was tested with budesonide found 3·2% positive in 144 patients.[16] Budesonide has been available in the United Kingdom since 1983 as an inhaler (Pulmicort®), and since 1984 as a nasal spray (Rhinocort®). However, a topical preparation (Preferid®) has only been available since 1989. Of the seven patients in this series who were allergic to budesonide six had not been exposed to any of the above formulations. The seventh presented with acute eczema within days of the first use of Preferid® cream. It therefore seems probable that in all our cases budesonide is cross-reacting with other steroids, e.g. hydrocortisone or hydrocortisone 17-butyrate.

In considering those 12 patients who were negative to TP but positive to another steroid, no single steroid which was tested would have been useful for screening, but hydrocortisone 17-butyrate was positive in four. At present, a combination of TP and hydrocortisone 17-butyrate can be recommended for screening for steroid allergy—budesonide revealed no cases which would not have been detected in this way. However, eight cases (6·1%) in this series would have been missed by this approach, and there is a need for further markers of corticosteroid allergy.

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British Journal of Dermatology (1992) 127, 501–504.

Sustained renal function loss in psoriasis patients after withdrawal of low-dose cyclosporin therapy

M.J.KORSTANJE,* H.J.G. BILO† AND T.J.STOOF‡

**Department of Dermatology, University Hospital, Leiden, The Netherlands*
‡Department of Dermatology, Free University Hospital, Amsterdam, The Netherlands
†Department of Internal Medicine, 'De Weezenlanden' Hospital, Zwolle, The Netherlands

Accepted for publication 7 May 1992

Summary

Eight patients with psoriasis received low-dose cyclosporin (CyA) treatment for an average period of 12 months (range 4–16 months). There was great variability in minimal effective CyA dose. In 50% of the patients long-term treatment was limited by dose reductions necessitated by side-effects.

A considerable impairment of renal function during CyA therapy was found. Glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were measured with ^{125}I-iothalamate and ^{131}I-hippuran, respectively. Both at the end of the active treatment period (GFR-CyA and ERPF-CyA), and 4 months after withdrawal of CyA (GFR-4mo and ERPF-4mo), there was sustained renal impairment: GFR-BL=97 (64–117), GFR-CyA reduction 17·8% (2·2–31·9%) [$P<0·02$], GFR-4mo reduction=9·8% (5·5–21·5%) ml/min/1·73 m^2 ($P<0·05$ vs. BL); ERPF-BL=401 (232–607), ERPF-CyA reduction=10·1% (7·4–27·3%) [$P<0·05$], ERPF-4mo reduction=13·5% (3·0–32·9%) ml/min/1·73 m^2 ($P<0·02$). Further studies of the effects on renal function during, and after, long-term therapy of psoriasis with low-dose CyA are warranted.

Several studies have shown a beneficial effect of cyclosporin (CyA) in patients with psoriasis.[1,2] Subsequent withdrawal of the drug, however, often leads to reappearance of the skin lesions to the same extent as before treatment. Therefore, treatment with CyA has to be continued according to the natural course of the disease. Withdrawal of CyA or dose reductions are often necessary because of the side-effects.[3,4] It has been suggested that side-effects are completely reversible after withdrawal of CyA, but there are reports of chronic irreversible renal injury with a CyA dose as low as 4·6 mg/kg/day.[5,6] Morphological renal changes were also found in a patient treated for psoriasis with a dose of 2·5 mg/kg/day.[7]

We carried out a pilot study with low-dose CyA for psoriasis, in order to determine whether renal impairment is completely reversible after withdrawal of CyA.

Methods

Eight patients with chronic plaque psoriasis (median age 39 [range 25–60] years; median duration of disease 17 [range 4–43] years) were studied. The median psoriasis area and severity index (PASI)[8] score was 19·8 (range 18·0–23·1). Patients with concomitant disease, proteinuria, or haematological or biochemical measurements outside the normal range, were excluded.

All patients received 5·0 mg/kg/day CyA given in two doses. After week 12 the CyA dose was reduced 0·35 mg/kg/day at monthly intervals. The CyA dose associated with the first rise in PASI score was regarded as the minimal effective dose. In the event of relapse (PASI above 50% baseline value) CyA was withdrawn. In any case CyA was withdrawn after 16 months of therapy.

If there was a rise in serum creatinine of more than 30% over baseline, a rise in serum potassium above the upper limit of the normal range, or a diastolic blood pressure above 95 mmHg on two consecutive visits, the CyA was reduced by about 25%. If the abnormality was not corrected within 4 weeks, a further reduction by 25% was made.

Whole blood CyA levels were initially checked weekly during the first month of treatment and every 4 weeks thereafter. Trough CyA concentrations (ng/ml) were measured 12–14 h after the last dose of CyA using a monoclonal antibody RIA-kit (Sandoz Ltd, Basle, Switzerland). Before initiating CyA treatment (baseline=BL),

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Correspondence: Dr M.J.Korstanje, Department of Dermatology, University Hospital Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

after 12 weeks of therapy, and 4 weeks after complete withdrawal of CyA, renal function tests were performed using simultaneous determinations of the ^{125}I-iothalamate and ^{131}I-hippuran clearances for glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), respectively.[9] The GFRs and ERPFs were normalized for body surface area. Clearances were expressed as ml/min/1·73 m^2. The GFR and ERPF were performed at 09.00 a.m. after the patient had fasted for at least 9 h.

Renal blood flow (RBF) was calculated by:

$$\text{RBF} = \frac{\text{ERPF}}{\text{1-haematocrit}},$$

and expressed as l/min. Blood pressure was measured in a sitting position, after 5 min rest, using a standard mercury sphygmomanometer. Total renal vascular resistance (TRVR) was calculated by:

$$\text{TRVR} = \frac{\text{MAP}}{\text{RBF}} \times 80$$

and expressed as dyn s/cm^5 (MAP = mean arterial pressure = diastolic BP + [pulse pressure/3]). Haematological and biochemical measurements were performed using standard laboratory techniques.

Statistical analysis

All results are expressed as medians and ranges. For statistical analysis, a two-tailed Wilcoxon signed-rank test for paired observations was used. A *P*-level of $<0·05$ was considered significant.

Results

The results are listed in Table 1. The psoriasis improved considerably in all patients. The patients continued therapy for 4–16 months, (median 11·8 months). Only four patients continued therapy for 16 months. The other four patients discontinued therapy earlier because of a relapse in psoriasis, as a result of the reduction in CyA dose. The minimal effective dose was 3·2 (2·3–4·2) mg/kg/day. Four patients had a 25% dose reduction because of side-effects, mainly a rise in serum creatinine above 30% of baseline. The renal parameters and mean renal parameters are listed in Tables 2 and 3, respectively. After 12 weeks of therapy we observed, in relation to baseline, a reduction in GFR of 17·8% (2·2–31·9%), ERPF 10·1% (7·4–27·3%), RBF 6·0% (4·1–17·5%), and an increase in TRVR of 15% (NS). There was limited improvement 4 months after withdrawal of CyA, GFR −9·8% (5·5–21·5), ERPF −13·5% (3·0–32·9%), RBF −7·7% (1·6–18·1%) and TRVR +17% (NS).

Discussion

CyA is effective in psoriasis but there appears to be great variability in the minimal effective dose (range 2·33–4·25 mg/kg/day). The median minimal effective dose of 3·2 mg/kg/day is comparable with values reported previously.[10,11]

In four patients long-term treatment efficacy was limited by dose reductions necessitated by side-effects. After dose reduction, the psoriasis relapsed. This indi-

Table 1. CyA therapy for psoriasis – long-term treatment results

Patient no.	Age (years)	PASI BL	BP BL	CyA level	Dose red.	PASI red.%	BP Wk12	CyA MED	CyA mo	CyA dose	CyA end level	BP end
1	33	18·7	125/80	115	—	100	120/80	2·33	16	2·33	<25	120/80
2	34	18·0	115/65	145	K+	62·2	110/60	4 05	4	4·05	60	115/65
3	25	21·0	135/85	135	Creat.	92·3	130/90	3·71	8	2·66	50	130/90
4	60	18·1	135/90	115	—	90·6	140/90	2·50	16	2·50	60	135/90
5	41	20·0	130/80	120	—	83·0	125/85	2·60	16	2·60	50	140/90
6	45	20·5	120/80	135	Creat	92·7	135/95	2·44	13	2·44	40	125/85
7	30	23·1	120/70	110	Creat.	69·0	120/70	4·25	5	4·22	85	120/70
8	44	19·0	135/85	155	—	98 9	130/80	4·30	16	2·50	35	135/80

BL, baseline; BP, blood pressure (mmHg systolic/diastolic); CyA level, the mean CyA trough blood level (ng/ml) during the first 12 weeks of treatment Dose red., dose reductions because of side-effects; PASI red., reduction of PASI at week 12 in comparison with baseline; CyA mo., CyA therapy in months until CyA was withdrawn; CyA MED, minimal effective dose of CyA (mg/kg/day); CyA dose, CyA dose (mg/kg/day) during the last month(s) of CyA therapy; CyA end level, the mean CyA trough blood level (ng/ml) in the last month of treatment; BP end, the blood pressure in the last month of treatment (mmHg).

Table 2. Renal parameters

	Baseline				Week 12				4 months after CyA withdrawal			
Patient no.	Creat.	GFR	ERPF	TRVR	Creat.	GFR red.%	ERPF red.%	TRVR % increase	Creat.	GFR red.%	ERPF red.%	TRVR % increase
1	98	91	374	41·6	100	2·2	5·1	−1·5	100	+5·5	12·8	8·1
2	90	112	522	25·1	102	20·5	7·3	−12·4	98	21·4	23·4	7·1
3	77	117	604	24·1	100	23·9	27·3	18·0	86	5·1	24·2	16·2
4	96	83	320	50·6	96	10·8	3·1	1·0	99	13·3	2·5	−1·2
5	92	88	368	41·3	104	19·3	12·2	19·8	108	19·3	32·9	52·0
6	95	64	232	41·8	105	12·5	+7·4	44·0	104	1·6	+3·0	46·0
7	87	116	607	18·2	100	31·9	18·1	48·1	96	11·2	10·7	15·3
8	84	102	428	36·8	106	21·6	14·9	3·2	91	11·7	4·2	−8·0

Creat., serum creatinine (μmol/l); GFR, glomerular filtration rate (ml/min); ERPF, effective renal plasma flow (ml/min); TRVR, total renovascular resistance (1000 × dyn s/cm^5).

cates that the therapeutic window for CyA therapy is small.

It is assumed that after low-dose CyA therapy for psoriasis all side-effects are reversible. However, few studies have been published in which renal function was studied with adequate GFR and ERPF measurements. Often GFR was measured after 8 or 12 weeks of therapy and not thereafter.[12] To our knowledge, only one study has been published in which the reversibility of the observed renal impairment in low-dose CyA therapy for psoriasis has been adequately studied.[13] In this study by Powles *et al.*[13] it was shown that impairment of renal function is reversible to a large extent after long-term therapy with CyA. However, there were no baseline values for GFR and ERPF available. Therefore, an irreversible component of the renal impairment cannot be reliably excluded in their study design. Irreversible renal damage with long-term low-dose CyA therapy has been reported in patients with conditions other than skin diseases,[5,6] and morphological renal changes have been observed in psoriasis patients treated with low-dose CyA therapy for 6–18 months.[7] Morphological changes were detected in a patient treated with a dose as low as 2·5 mg/kg/day.[7]

Table 3. Mean renal parameters

Baseline (BL)	Week 12	4 months after CyA withdrawal
Creat. 89·9	102 (*P*<0·02 vs. BL)	97·8 (NS)
GRF 96·6	78·4 (*P*<0·02 vs. BL)	86·8 (*P*<0·05 vs. BL)
ERPF 432	378 (*P*<0·05 vs. BL)	367 (*P*<0·02 vs. BL)
TRVR 34·9	39·7 (NS)	41·1 (NS)

Creat., serum creatinine (μmol/l); GFR, glomerular filtration rate (ml/min); ERPF, effective renal plasma flow (ml/min), TRVR, total renovascular resistance (1000 × dyn s/cm^5); NS, not significant.

The patients in our study were treated with an initial dose of 5 mg/kg/day. After week 12 the CyA dose was subsequently reduced until the minimal effective dose was reached, and thereafter the patients were treated with a minimal effective dose of CyA (median 3·2; range 2·33–4·25 mg/kg/day). We found considerable renal impairment, as measured by GFR and ERPF, at week 12. It is possible that, with a lower initial dose of CyA, the renal impairment would have been less.

Four months after withdrawal of CyA sustained renal impairment could be demonstrated, in some patients as high as 20% reduction from baseline value. It is possible that renal function might improve further after this period, but until this has been investigated, CyA should be used with caution in dermatology, particularly as irreversible morphological renal changes have been observed in psoriasis patients.[7] Functional renal changes should be kept to a minimum, and an increase in serum creatinine to more than 30% over baseline should not be allowed to occur.[14]

Further studies are warranted, in which the renal side-effects during and after long-term CyA therapy are monitored.

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British Journal of Dermatology (1992) 127, 505–508.

Nine years' experience of BELD combination chemotherapy (bleomycin, vindesine, CCNU and DTIC) for metastatic melanoma

G.I.STABLES, V.R.DOHERTY* AND R.M.MACKIE

University Department of Dermatology, Western Infirmary, Glasgow G11 6NT, U.K.

**Department of Dermatology, Victoria Hospital, Kirkcaldy, KY2 5AH, U.K.*

Accepted for publication 12 June 1992

Summary

During the 9-year period from 1982 to 1991, 72 patients with melanoma were treated with a 5-day quadruple drug chemotherapy regime (BELD) comprising bleomycin, vindesine (Eldesine®), CCNU (Lomustine®) and DTIC. Forty-three patients had stage III melanoma, 34 of whom had evaluable disease. Of these 34, six (17·6%) achieved a complete response (CR), eight (23·5%) had a partial response (PR), five (14·7%) had stabilized disease (SD) and 15 (44·1%) had progressive disease (PD). Overall median survival of stage III melanoma patients was 38 weeks. Median survival of responders (CR+PR) was 47 weeks and 21 weeks for non-responders (SD+PD) ($P<0{\cdot}005$). Median follow-up time was 38 weeks. Following these encouraging results, 30 patients with stage II melanoma received BELD chemotherapy as adjuvant therapy after regional node dissection and clearance. Adjuvant BELD chemotherapy did not alter survival in these patients. BELD combination chemotherapy is well-tolerated, the main problems being nausea, vomiting, and leucopenia. We have maintained a combined response rate (CR+PR) of 41·1% for stage III disease. This is comparable with other combination chemotherapy regimes, which have as yet not been superseded by the newer biological therapies.

The prognosis of metastatic melanoma is poor. The most widely used single cytotoxic agent is DTIC (dimethyl-triazeno-imidazole-carboxamide) with reported response rates in disseminated melanoma of 20–25%.[1] Other classes of cytotoxics of proven activity are the vinca alkaloids, the nitrosoureas and cisplatin. Higher response rates have been reported using combination chemotherapy. A combined response rate of 40% was reported using bleomycin, vincristine, CCNU and DTIC (BOLD).[2] Vincristine as a single agent has a reported response rate of 20%,[3] whereas vindesine as a single agent has a higher reported response rate, as confirmed by a Scottish Melanoma Group study which reported a response rate of 26%.[4] This led us to substitute vindesine for vincristine to produce the BELD regime. In a pilot study of BELD combination chemotherapy in stage III melanoma (presence of metastases distant from regional lymph nodes) in 20 patients we reported a combined response rate of 45%.[5] This encouraged us to continue using BELD in stage III disease, but also to begin a pilot study of BELD chemotherapy in an adjuvant setting for patients with stage II disease (metastatic disease in regional lymph nodes) after lymphadenectomy. We present here our cumulative experience of BELD chemotherapy for stage III and stage II melanoma over a 9-year period.

Methods

In the period from May 1982 to April 1991, 72 consecutive patients with metastatic melanoma, who had a life expectancy of more than 6 weeks, were treated with BELD chemotherapy: 42 patients (25 male, 17 female) with stage III melanoma and 29 patients (16 male, 13 female) with stage II disease. One additional male patient, initially treated with BELD for stage II disease, subsequently developed stage III disease and was treated with a second course of BELD chemotherapy. Histological proof of metastatic melanoma was obtained in every case.

All patients had two or more cycles of chemotherapy, 4–6 weeks apart. Haemoglobin, leucocyte and platelet counts were checked before, during and after each cycle.

Correspondence: Dr G.I.Stables.

BELD combination chemotherapy is a quadruple drug regime combining bleomycin, vindesine (Eldesine®), CCNU (Lomustine®) and DTIC (dimethyl-triazeno-imidazole-carboxamide). Bleomycin is given on days 1 and 4 at a dose of 7·5 mg for the first cycle and 15 mg for subsequent cycles of chemotherapy. It was originally administered subcutaneously, but because some patients developed tender, subcutaneous nodules, a recognized complication of subcutaneous administration, it was subsequently given intravenously. Vindesine is given intravenously at a dose of 5 mg on days 1 and 5. Lomustine is given orally on day 1 at a dose of 80 mg/m^2 to a maximum single dose of 150 mg. DTIC is given as an intravenous infusion at a dose of 200 mg/m^2, to a maximum dose of 400 mg, on each of the 5 days.

Patients with stage III disease were evaluated before starting treatment and after each set of two cycles of chemotherapy. Evaluation of disease was by clinical examination, regular chest X-ray, and hepatic ultrasound. In addition, since 1983 all patients have been evaluated by computed tomography (CT) scan. Isotope brain scans were used before CT was available, and isotope bone scans were used when clinically indicated.

Patients were evaluated using the following generally accepted criteria: complete response (CR), the disappearance of all evidence of disease, lasting at least 2 months; partial response (PR), a decrease of at least 50% in tumour volume, again lasting for more than 2 months; stable disease (SD), a change of less than 50% in tumour volume; progressive disease (PD), an increase of more than 50% in tumour measurements, or the appearance of new lesions.

Results

There were 43 stage III patients, median age 53 (23–73). Thirty-seven patients had previous regional node dissection and clearance and/or excision of subcutaneous metastases; five patients had previous isolated limb perfusion; five patients had a previous 6-week course of weekly intravenous vindesine (as part of a Scottish Melanoma Group multicentre trial[4]); three patients had received radiotherapy to bone, brain and soft tissue metastases; one patient had a single brain metastasis excised, and one patient, as already mentioned, had previous BELD chemotherapy for stage II disease. Twenty-eight patients had two cycles, four had three cycles, four had four cycles, two had five cycles, three had six cycles, one had seven cycles and one patient had 10 cycles of chemotherapy. Following BELD chemotherapy, two patients had isolated limb perfusion, four patients had radiotherapy (two to bone, one to brain and one to soft tissue metastases) and four patients received alternative chemotherapy.

Overall median survival of stage III patients was 37 weeks (8–440). Thirty-four patients had evaluable disease, and nine non-evaluable. Of the 34 with evaluable disease, six (17·6%) had a complete response (CR), achieved in two cycles for four patients and six and 10 cycles of chemotherapy in the other two CR patients, eight (23·5%) had a partial response (PR), five (14·7%) had stabilized disease (SD) and 15 (44·1%) had progressive disease (PD). This gives a combined response rate (CR+PR) of 41·1%. Median survival of responders (CR+PR) was 47 weeks and for non-responders

Table 1. Side-effects and complications experienced during treatment with BELD chemotherapy

Vomiting	55 (76·4)
Vomiting day 1 only	38 (52·8)
Leucopenia (WHO grade 2 and 3[6])	35 (48·6)
Thrombocytopenia (WHO grade 2 and 3[6])	6 (8·3)
Modification of dose because of leucopenia and/or thrombocytopenia	7 (9·7)
Bleomycin pyrexia	38 (52·8)
Peripheral paraesthesia attributed to vindesine	7 (9·7)
Dystonic reaction to high-dose metoclopramide	2 (2·8)
Septicaemia	1 (1·4)
Deep venous thrombosis and pulmonary embolus	1
Recurrence of old tuberculosis	1
Urticaria	1
Photosensitivity	1

* Numbers in parentheses are percentages.

(SD+PD) was 21 weeks ($P<0{\cdot}005$). Median follow-up time was 37 weeks. Nine females responded, compared with five males, and median survival for females was 38 weeks compared with 28 weeks for males, suggesting that females respond better and survive longer, although the differences were not statistically significant. The sites of metastases which responded to BELD chemotherapy were pulmonary, nodal and subcutaneous, rather than bone and brain. The median duration of response for complete responders was 23+ weeks and for partial responders was 8 weeks.

All 30 stage II patients, median age 46 (22–71), had previous regional node dissection and clearance. They all had two cycles of BELD chemotherapy. Six patients received subsequent treatment for recurrence and progression of disease: three patients received alternative chemotherapy; two patients received radiotherapy for cerebral metastases, and one patient had a single cerebral metastasis excised. Overall median survival of stage II patients (excluding the patient who went on to further BELD chemotherapy for stage III disease) was 90 weeks (24–380).

The side-effects and complications experienced by our patients are shown in Table 1. The BELD chemotherapy regime is highly emetogenic, although in over two-thirds of patients vomiting occurred on day 1 of the chemotherapy cycle only. In those patients whose nausea and vomiting is not controlled by antiemetic regimes such as metoclopramide, prochlorperazine, high-dose metoclopramide, dexamethasone and lorazepam, used either individually or in various combinations, ondansetron, the specific 5HT-3 antagonist, is effective.[7] Leucopenia and thrombocytopenia were significant problems but the dose of chemotherapy required modification in only seven patients. About 50% of patients experienced a bleomycin reaction, which was sometimes distressing, with fever and rigors occurring on the days of bleomycin administration. One patient developed repeated photosensitivity with each cycle of chemotherapy, a recognized but rare side-effect of DTIC.

Discussion

The response of advanced melanoma to systemic treatment, whether with chemotherapeutic agents, biological therapy, hormonal therapy, or any other drug, is generally disappointing. The single most effective chemotherapeutic agent is DTIC. Other single agents of proven efficacy are the vinca alkaloids and nitrosourea group of drugs. The newest derivative of the nitrosourea group is fotemustine, with a reported overall response rate of 24%, but a 25% response rate in patients with cerebral metastases.[8] Cisplatin has a reported response rate of up to 25%.[9] Bleomycin alone has no proven clinical activity against melanoma.

Combination chemotherapy regimes have produced better response rates than single agents, Most regimes have been based on DTIC, such as BOLD, with a reported response rate of 40%.[2] When modified to produce the BELD regime, we reported a response rate of 45% in a pilot study.[5] Cisplatin combined with vinblastine and bleomycin has produced a similar response rate of 43%.[10] The combination of DTIC, vindesine and cisplatin produced a response rate of the same order (41%).[11]

The encouraging results from our initial pilot study of the use of BELD combination chemotherapy led to its continued use in stage III patients. Over a 9-year period we have maintained an overall combined response rate of 41·1%, with responders surviving significantly longer than non-responders. Females appeared to respond better and survive longer than male patients, and soft tissue and pulmonary metastases respond better than bone and cerebral metastases, features in agreement with previous observations.[12]

Occasional reports of spontaneous regression of melanoma, both primary and secondary, histological evidence of tumour regression, and the discovery of melanoma-specific cytotoxic T-cells, have suggested the importance of the host immune response to melanoma. This has led to intense interest in the interferons, interleukin 2 (IL-2) alone and IL-2 with lymphokine-activated killer (LAK) cells or tumour-infiltrating lymphocytes (TILs). Of particular interest is the fact that a small group of patients treated with these agents have experienced long-term remissions.[12]

Interferon-α, -β and -γ have all been shown to have some effect on metastatic melanoma, with reported response rates of between 5 and 25%.[13] Recently, a combined response rate (CR+PR) of 70% was reported following the use of interferon-α2b in cutaneous metastatic melanoma.[14] Trials with combinations of interferons and conventional chemotherapeutic agents are currently in progress.

IL-2 induces the generation of antigen-specific cytotoxic T cells, and increases natural killer cell activity. When IL-2 is used in combination with lymphokine-activated killer cells, a response rate of 22% has been reported.[15] The combination of IL-2 with autologous tumour-infiltrating lymphocytes produces a similar response rate, although it is not possible to isolate and culture TILs in over half of patients.[16] In an attempt to reduce toxicity and improve efficacy IL-2 is being combined with interferon and conventional chemotherapeutic agents. However, the early reports on small numbers of patients are difficult to evaluate. Currently being developed are tumour vaccines aimed at stimulating host immune response against melanoma antigens, and monoclonal antibodies against melanoma-associated antigens.[17]

Stage II melanoma patients present a very difficult management problem with regard to treatment and prevention of recurrence of metastatic melanoma. Following initial encouraging results of BELD chemotherapy in stage III disease, a group of stage II melanoma patients were treated with two cycles of adjuvant BELD chemotherapy. In a review of 142 patients undergoing therapeutic lymphadenopathy for clinical stage II malignant melanoma in the west of Scotland, survival was not altered in the 25 patients who received two cycles of adjuvant BELD chemotherapy, and the overall 5-year survival reported was 26%.[18] There has, as yet, been no evidence of any benefit of adjuvant chemotherapy, biological therapy, or both.

Until the newer approaches to therapy have been fully evaluated, BELD combination chemotherapy can produce respectable response rates of significant duration, with the added advantage of a low, acceptable level of toxicity.

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British Journal of Dermatology (1992) 127, 509–512.

Treatment of psoriasis with a 311-nm UVB lamp

E.PICOT, L.MEUNIER, M.C.PICOT-DEBEZE,* J.L.PEYRON AND J.MEYNADIER

Department of Dermatology (Pr Meynadier), University Hospital, Saint-Charles Hospital, 34059 Montpellier Cedex, France
**Department of Medical Information, University Hospital, Lapeyronie Hospital, 34059 Montpellier Cedex, France*

Accepted for publication 10 March 1992

Summary In a left–right comparative study, the Philips TL-01 sunlamp, a new UVB fluorescent lamp, was evaluated in 15 patients with symmetrical psoriasis. One half of the body was treated in a cabin containing TL-01 lamps, and the other half in a cabin containing TL-12 lamps. The patients were treated three times/week, and the study was conducted in a randomized, double-blind fashion. The percentage response of psoriatic lesions was determined on the tenth and twentieth exposures. The therapeutic effect of the TL-01 lamps was superior to that of the TL-12 lamps, and treatment was better tolerated, particularly with regard to episodes of burning. This new lamp appears to provide more effective and safer phototherapy for psoriasis.

UVB phototherapy has an established role in the treatment of psoriasis and is still being improved. The first experiments with phototherapy were carried out by Alderson in the 1920s,[1] and confirmed by Goeckerman.[2] The effectiveness of UVB, when not combined with a photosensitizing agent, was originally questioned by Epstein,[3] and more recently by Young,[4] but results were imprecise because of the radiometric and dosimetric parameters used. However, in 1976 Fischer[5] demonstrated the effectiveness of UVB alone in the treatment of psoriasis, and this was confirmed by Parrish.[6,7] The study of Parrish and Jaenicke[7] in 1981 still constitutes the most precise information available on the therapeutic action spectrum in psoriasis. It revealed that UVC and short wavelength UVB are ineffective, and that the most beneficial wavebands in psoriasis are situated near 313 nm (Fig. 1). In view of these results, Van Weelden and Van Der Leun[8] proposed the development of a light source excluding shorter wavelength, erythemogenic UVB.

The Philips TL40W/01 lamp, a new fluorescent lamp, which produces a peak narrow-band emission at 312 nm and a minor peak at 305 nm (Fig. 2), should therefore be theoretically more effective in the treatment of psoriasis than the Philips TL40W/12 lamp, which is a conventional broad-band UVB lamp emitting a considerable amount of irradiation in the wavelength range below 300 nm (Fig. 3). We attempted to confirm the superiority of the TL-01 lamp in a comparative study.

Correspondence: Dr E.Picot

Methods

The study was carried out at Montpellier (France) from January to June 1990. Twenty-one patients were enrolled, but only 15 completed the study (seven females, eight males; age range 24–81 years, mean 46·5). Informed consent was obtained from all patients. Two patients defaulted without notification, and four for personal reasons.

Patients had plaque-type (6), plaque/guttate (5) or guttate psoriasis (4), which was widespread and symmetrical. The mean duration was 11·8 years (range 4 months–28 years). Patients with a history of photoaggravated psoriasis were excluded. None had received UVB treatment, photochemotherapy (PUVA) or retinoids for 3 months prior to the study. All photosensitizing medical treatment, and all topical treatment, apart from pure vaseline or 1% salicylic acid in petrolatum was prohibited.

Light sources

Twelve Philips TL40W/01 lamps were installed in an ordinary UVB cabin provided by Dixwell Laboratory (17–19 rue Mazagran, 69007 Lyon). The average irradiance was 1·4 mW/cm^2 measured by a calibrated UVB meter (Photometer type DF series DFX Silicon photovoltaic detector).

Twelve Philips TL40W/12 lamps were placed in an identical cabin. The average irradiance in this cabin, measured by the same UVB meter, was 2·1 mW/cm^2.

The irradiances were checked with a Waldmann AG UVB detector (Schwenningen, Germany).

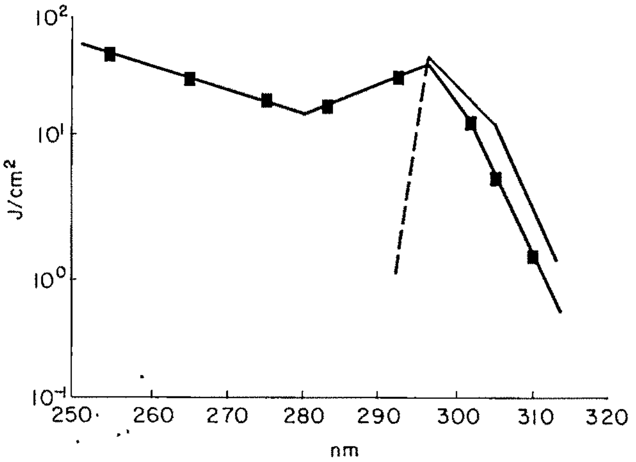

Figure 1. Erythema action spectrum mean (—■—); phototherapy action spectrum mean (——); (– – –) shows the steep decline in therapeutic effectiveness at wavelengths less than or equal to 290 nm.

Treatment schedule

One vertical half of the body, chosen randomly, was treated in the cabin containing the TL-01 lamps, and the other half treated in the cabin containing TL-12 lamps. During irradiation of one half of the body, the other half was shielded by a thick material preventing penetration of the UV rays (photometric verification).

A conventional therapeutic schedule was employed in treatment with the TL-12 lamps. The first exposure was 70% of the average MED estimated from the patient's skin type (average MED of skin type II, 80 mJ/cm²; III, 120 mJ/cm²; IV, 150 mJ/cm²). Subsequent doses were given on the basis that, if the previous exposure had induced no perceptible effect, the exposure time was increased by 40%. If there was slight erythema, the exposure time was increased by 20%, and if marked erythema occurred, the same exposure time was used again. Doses were decreased by 20% in cases of minor burning, and by 40% in cases of mild burning. Treatment was temporarily stopped if severe burning occurred.

For treatment with TL-01 lamps, the initial exposure dose was identical to that used in the TL-12 cabin. The same exposure schedule was employed. In this way the applied doses in each cabin were not associated with the same risk of erythema, and were not therefore equierythemogenic. In fact, the erythemogenic doses of the TL-01 lamps were 4–6 times longer than those of the conventional UVB phototherapy lamps.[9,10] The maximum

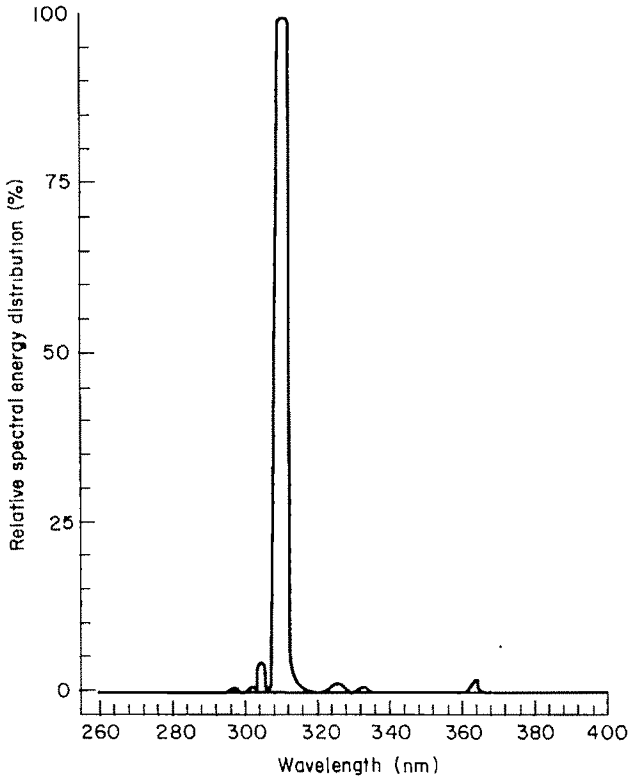

Figure 2. Relative special energy distribution of the Philips TL-01 fluorescent lamp.

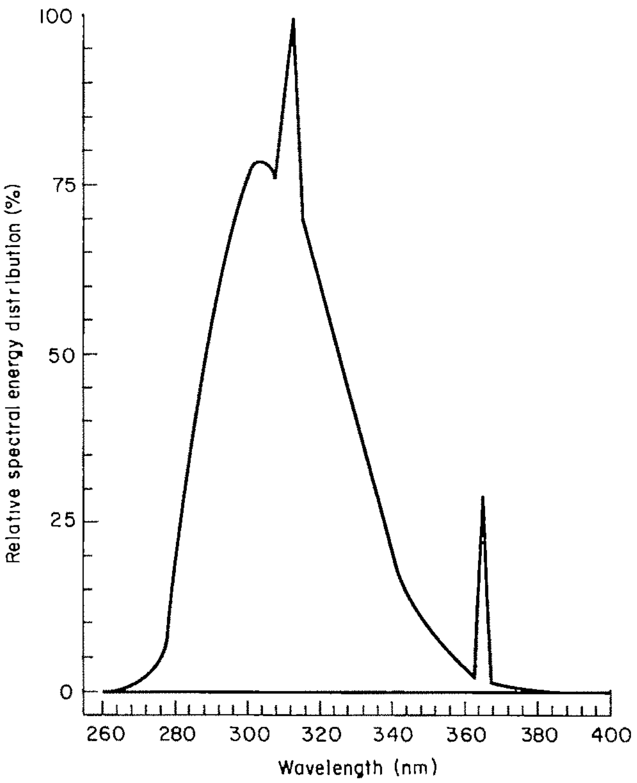

Figure 3. Relative spectral energy distribution of the Philips TL-12 fluorescent lamp.

exposure time of each half of the body was fixed at 16 min. The patients were treated three times/week for a maximum of 10 weeks.

Therapeutic evaluation

An assessment of erythema, scaling, infiltration and extent of the lesions was carried out prior to treatment. The intensity of erythema, desquamation and infiltration was rated according to the following scale: 3, severe; 2, moderate; 1, mild; 0, absent. In addition the percentage of involved surface area was recorded. The severity of disease was evaluated by means of the psoriasis area and severity index (PASI) score based on the above parameters. An initial score of the severity of psoriasis was thus determined (S_0).

The therapeutic effects were assessed at the tenth and twentieth exposure (S_{10} and S_{20}) by another experienced clinical observer using the same scoring system. This observer had no knowledge of which therapy was administered to which side of the patient. On the tenth and twentieth exposure the percentage response of psoriatic lesions was determined:

$$\left(\frac{S_0 - S_{20}}{S_0} \times 100\right).$$

At the time of the last exposure, patients were asked about their preference with regard to effectiveness.

Adverse effects such as burning, pigmentation or pruritus were evaluated by semiquantitative scoring (0, no symptoms; 1, minimal symptoms; 2, moderate; 3, severe symptoms).

Statistical analysis

All analyses were carried out with a VAX/VMS statistical package, using the BMDP programme. The Wilcoxon test was adapted for this study.

Results

The percentage overall improvement for the TL-01 and TL-12 lamps at the twentieth exposure were 78·5 and 73·9%, respectively. Hence, psoriasis cleared to a greater extent with TL-01 lamps than with TL-12 lamps, and this difference was significant ($P<0{\cdot}05$). No significant difference between the two lamps was found at the tenth exposure (Table 1). In seven patients the TL-01 lamps gave superior results, in one patient the TL-12 lamps were more effective, and there was no significant difference in the remaining seven patients.

Table 1. Comparison of UVB phototherapy with Philips TL-01 and TL-12 lamps

	TL-01	TL-12	P
Mean S_0	27·9	27·6	NS
Mean S_{10}	12·9	12·5	NS
Mean S_{20}	6·6	7·8	<0·01
% mean response of psoriasis $\left(\frac{S_0 - S_{20}}{S_0} \times 100\right)$.	78·5	73·9	<0·01
Mean total cumulative dose at the twentieth exposure (J/cm²)	15·5	7·6	—
Mean score burning	0·33	2·1	<0·001
Mean score pigmentation	1·46	1·60	NS

The mean total cumulative dose of UVB was $15{\cdot}1 \pm 3{\cdot}8$ J/cm² (range 8·7–21·7 J/cm²) for the TL-01 lamps and $7{\cdot}6 \pm 4{\cdot}2$ J/cm² (range 2·14 – 14·5 J/cm²) for the TL-12 lamps.

Adverse effects were assessed separately. Pigmentation was practically identical with both lamps. Burning episodes occurred less frequently with the TL-01 lamps, and the difference was significant ($P<0{\cdot}001$). Itching did not occur after use of either lamp.

Twelve patients preferred TL-01 therapy, and three had no preference.

Discussion

In this study, UVB phototherapy using TL-01 lamps was more effective than treatment with conventional UVB TL-12 lamps. These findings are supported by previously published studies[9,11–13] which showed superior efficacy of the TL-01 lamps with equierythemogenic schedules. Doses from the TL-01 lamps were therefore multiplied by a factor of 4–6 compared with the TL-12 lamps. Our study is the first using a non-equierythemogenic schedule, and we established that the TL-01 lamps were effective with an infra-erythemogenic regime.

At the twentieth exposure the mean total cumulative dose from TL-01 lamps received by our patients was approximately twice that from the TL-12 lamps. This is because of the rarity and mildness of episodes of erythema caused by TL-01 lamps, which allowed a regular progression of doses up to 40%. TL-12 lamps often produced erythema, and therefore the progression with these lamps was slower and less regular.

No moderate or serious burning occurred with TL-01 lamps while using this therapeutic schedule. Only minor burning was noticed. On the other hand, episodes of burning with the TL-12 lamps were relatively common. This short-term innocuousness of TL-01 lamps results in better treatment compliance, and the avoidance of a possible Koebner phenomenon. It also explains the patients' preference. In addition, TL-01 lamps appear not only safer in the short term, but probably also in the long term. A study[13] carried out on animals revealed that TL-01 lamps were slightly less carcinogenic than TL-12 lamps, using an equierythemogenic schedule. However, a recent study[14] produced contrary results. Differences in several parameters make comparison difficult, but we are surprised that in the study by Flindt-Hansen *et al.*[14] the ratio of the MED of mouse skin between the two lamps was 11, compared with 5·8 in the study by Van Weelden *et al.*[13] Although further studies may be necessary, the suppression of short-wavelength UVB has the theoretical advantage of being less carcinogenic.

We have shown that the Philips TL-01 lamps have superior efficacy and tolerance. Further studies will be required to confirm these findings and to determine the optimum therapeutic schedule, but our results appear very convincing. Another recent study[10] concluded that phototherapy with narrow-band UVB is as effective as photochemotherapy (PUVA), which is more complicated and inconvenient.

The value of this new therapy should be assessed in other dermatoses where conventional broad-band UVB phototherapy is effective, for example, in atopic dermatitis, severe pruritus associated with chronic renal failure, polymorphic light eruption, and solar urticaria.

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British Journal of Dermatology (1992) **127**, 513–518.

Treatment of cutaneous lupus erythematosus with acitretin and hydroxychloroquine

T.RUZICKA,* C.SOMMERBURG,† G.GOERZ,‡ P.KIND‡ AND H.MENSING§

*Departments of Dermatology, Universities of *München, ‡Düsseldorf and §Hamburg, Germany*
†Department of Biometrics, STATICON GmbH, Planegg, Germany

Accepted for publication 21 April 1992

Summary A randomized, double-blind, multicentre study was performed to compare the efficacy of acitretin (50 mg/day) with hydroxychloroquine (400 mg/day) in 28 and 30 patients, respectively, suffering from cutaneous lupus erythematosus (LE). The study was carried out over an 8-week period. Improvement of facial LE lesions after treatment with acitretin and hydroxychloroquine was assessed using several clinical parameters. In the acitretin group there was marked improvement or clearing of erythema in 10/24 patients (42%), of infiltration in 15/24 (63%) and of scaling/hyperkeratosis in 12/20 (60%). In the hydroxychloroquine group there was complete clearing or marked improvement of erythema in 17/25 patients (68%), of infiltration in 17/25 (68%) and of scaling/hyperkeratosis in 15/23 (65%). Overall improvement occurred in 13/28 patients (46%) treated with acitretin and in 15/30 patients (50%) with hydroxychloroquine. The incidence of side-effects was higher in the acitretin group, and necessitated discontinuation of treatment in four patients. The present results demonstrate that both acitretin and hydroxychloroquine provide effective treatment in approximately 50% of cases of cutaneous LE.

A limited number of drugs are available for the treatment of chronic discoid lupus erythematosus (DLE) and subacute cutaneous LE (SCLE).[1–5] Their use is restricted by poor efficacy in a large proportion of patients and/or side-effects and contraindications. Apart from corticosteroids, the most frequently employed drugs are the antimalarials chloroquine and hydroxychloroquine,[4,5] but dapsone[6] and thalidomide[7] may also be effective. This list has recently been supplemented by the aromatic retinoids etretinate and acitretin,[8–10] which were shown in open, uncontrolled trials to lead to remission in a substantial number of LE patients. Because of a need for new drugs for the treatment of LE and the encouraging results of the pilot trials, it was considered necessary to evaluate the efficacy of the aromatic retinoids in a controlled trial, and to establish their relative value by comparison with an established therapeutic agent. Because acitretin, a drug used predominantly in the treatment of hyperkeratotic and inflammatory skin diseases such as psoriasis, shows a pharmacokinetic profile superior to etretinate (which, however, may be offset by the recent findings of retroconversion to etretinate in some patients), a randomized, double-blind study was performed to compare acitretin with hydroxychloroquine, a drug frequently used in the treatment of cutaneous LE. The present study was performed at three specialized LE clinics in university departments of dermatology. The results confirm the efficacy of acitretin in cutaneous LE, and suggest that retinoids may enlarge the therapeutic arsenal for this disease.

Methods

The present study was conducted in a randomized, double-blind, parallel fashion. Because of the low incidence of the disease, and in order to complete the trial within a reasonable period of time, only specialized LE clinics participated. A total sample size of 40 patients was initially planned, but 60 patients were eventually enrolled in the study. The study protocol was reviewed and approved by the Ethics Committee of the Ludwig-Maximilians-Universität München.

Patients

In-patients and out-patients of either sex, aged 18–75 years, suffering from localized or disseminated DLE, or

Correspondence: Thomas Ruzicka, Department of Dermatology, Ludwig-Maximilians-Universität, Frauenlobstrasse, 9–11, D-8000 München 2, Germany.

SCLE, were eligible for enrolment. Females of childbearing age were admitted only when a β-HCG pregnancy test was negative, and effective contraception was used for a period of 1 month before commencing therapy, and up to 2 months after cessation of therapy. Exclusion criteria were: systemic LE, LE profundus, pregnancy, lactation, serious kidney disease (serum urea $>18 \cdot 5$ mmol/l, serum creatinine >100 μmol/l, repeatedly abnormal urine sediment) and severe liver disease (bilirubin $>50\%$ above normal; serum aspartate and pyruvate aminotransferase, and gamma-glutamyl transpeptidase levels $>100\%$ above normal; alkaline phosphatase $>25\%$ above normal), metabolic disorders requiring medical treatment (such as primary or secondary hyperlipoproteinaemias and diabetes), symptomatic gout, glaucoma, severe cardiac disease, haemopoietic disorders, retinopathy, epilepsy, glucose-6-phosphate dehydrogenase deficiency, hypersensitivity to 4-aminoquinoline, etretinate treatment during the previous 3 months, and any other systemic treatment within 4 weeks prior to the study. Written informed consent was requested and obtained from all patients.

Treatment

Patients were randomly assigned to receive either 50 mg acitretin (kindly provided by F. Hoffmann-La Roche) or 400 mg hydroxychloroquine daily over a period of 8 weeks. The medication was to be taken with breakfast (consisting of coffee or tea and at least one roll spread with butter). No other LE-specific systemic or topical treatment was permitted for the entire trial period, with the exception of emollients and sunscreens. Long-term medication for other diseases could be continued, provided that it had been taken for at least 4 weeks before the start of the study and was well tolerated, and that the dosage could be kept constant during the trial period.

Treatment was to be discontinued if patients withdrew consent, if severe side-effects occurred or abnormal laboratory values were observed, if another intercurrent acute disease occurred which required medication, if deterioration of LE and/or systemic involvement occurred, or if efficacy was not satisfactory after a treatment period of at least 4 weeks.

Assessment

Severity of disease was assessed using scores for erythema, infiltration and scaling/hyperkeratosis. An assessment was made at the initial visit using an ordinal scale (0 = absent, 1 = moderate, 2 = severe) and at biweekly follow-up visits. Complete clearing was graded as 0, improvement as 1 and no change or deterioration as 2. Further variables documented were the area of skin affected, clinical and laboratory findings, adverse events, LE-specific laboratory tests (C3, C4, antinuclear antibodies, anti-DNA antibodies, anti-Ro antibodies) before and after treatment, and blood count and urinalysis. Erythrocyte sedimentation rate, γ-glutamyl transpeptidase, serum glutamate oxalacetate transaminase, serum glutamate pyruvate transaminase, and total cholesterol and triglyceride levels were determined after 2, 4 and 6 weeks of treatment. After completion of the trial, an overall assessment of efficacy was carried out by the investigator using the following categories: complete clearing with or without central atrophy (90–100% improvement), marked improvement (50–89%), slight improvement (25–49%), no change ($<25\%$–0) and deterioration (<0).

Statistical analysis

The main objective of this study was to compare the efficacy of acitretin and hydroxychloroquine in the treatment of cutaneous LE, assuming that there was no difference between both treatments. Target variables were the effect on erythema, infiltration and scaling/hyperkeratosis on the face. Response for each of these was defined as complete clearing or marked improvement. To compare treatment effects, chi-squared tests were performed for the response rates. The aim of this approach was not to emphasize the equivalence of the treatments, but to generate hypotheses. This test is suitable for the comparison of frequencies of dichotomized responses between two samples. Because of the descriptive design of the study, each pairwise comparison was performed at the 5% level of significance. Additionally, 95% confidence intervals were calculated for the response rates within each treatment group as well as the difference of overall response rates between the treatment groups.

To compare changes in triglycerides within and between treatment groups, paired and unpaired *t*-tests were used. These tests are appropriate for continuous variables and for the comparison of mean effects in the same population at two points in time (paired *t*-test) or of independent populations at one point in time (unpaired *t*-test). Two-sided tests were used throughout. Mean values are presented with the standard deviation (SD) or with the standard error of the mean (SEM).

Both analyses of efficacy and tolerability were conducted as 'intent-to-treat', using a last-value analysis for drop-outs. Two patients (from the acitretin group), who were lost to follow-up after the initial examination, were excluded from both analyses.

Participating centres

The trial was conducted at three specialized LE clinics (Departments of Dermatology of the Universities of München, Hamburg and Düsseldorf). The trial period lasted from September 1987 until November 1989. Monitoring and statistical analysis were performed by STATICON GmbH, Planegg, Germany.

Results

Patient characteristics

Characteristics of the study population are summarized in Table 1. Discoid LE was the most common type of LE in each treatment group. Subacute cutaneous LE (SCLE) was more frequent in the hydroxychloroquine group. Thirteen patients of the acitretin group and 10 patients of the hydroxychloroquine group had already received LE-specific treatment. Pretreatment drugs administered in the acitretin group were antimalarials in nine patients, corticosteroids in two patients, and etretinate and sulphone in one patient each. In the hydroxychloroquine group eight patients received antimalarials, and one patient each received penicillin and etretinate. Pretreatment was effective in only three patients of each treatment group (one patient with etretinate and two patients with antimalarials in each group). Pretreatment had been discontinued at least 7 days before the beginning of the study in all but one case. This patient, who suffered from SCLE, received 8 mg methylprednisolone/day for 40 weeks before the beginning of the study. This drug was also administered throughout the entire trial period. However, improvement was only slight.

The pattern of LE localization was similar in both groups. The area predominantly involved was the face. Only four patients in the acitretin group and five patients in the hydroxychloroquine group had LE lesions on other body areas, i.e. scalp (two patients in the acitretin group and one patient in the hydroxychloroquine group), chest (two patients in the acitretin group and four patients in the hydroxychloroquine group), back (two patients in the acitretin group and four patients in the hydroxychloroquine group) and arms (one patient in the acitretin group and three patients in the hydroxychloroquine group). The distribution of the intensity of erythema, infiltration and scaling/hyperkeratosis showed a slight but not significant asymmetry. Infiltration was more pronounced in the hydroxychloroquine group and scaling/hyperkeratosis in the acitretin group (Table 2). Overall, both groups were regarded as comparable with respect to age, sex ratio, duration of LE and severity of LE lesions on the face.,

Premature cessation of treatment

Premature cessation, due to complete clearing before 8 weeks of treatment, was observed in the hydroxychloroquine group (three patients) only. Drop-outs in the acitretin group included four patients with adverse events, one patient with exacerbation and one patient due to non-compliance. In the hydroxychloroquine group there was only one drop-out due to lack of efficacy. The mean treatment period for acitretin was 52·5 days (SD = 16·4) and 56·0 days (SD = 9·4) for hydroxychloroquine.

Table 1. Patient characteristics

	Acitretin (%) $n=28$	Hydroxychloroquine (%) $n=30$
Demographic data		
Male	11 (39)	11 (37)
Female	17 (61)	19 (63)
Age (years)*	42·4 ± 14·2	44·1 ± 13·3
LE subset		
Discoid LE, localized	16 (57)	15 (50)
Discoid LE, disseminated	5 (18)	3 (10)
Subacute cutaneous LE	7 (25)	12 (40)

* Mean ± SD

Table 2. Severity of erythema, infiltration and scaling/hyperkeratosis of LE lesions (face only) in patients with cutaneous LE

	Acitretin (%) $n=24$	Hydroxychloroquine (%) $n=25$
Eythema		
Moderate	3/24 (13)	5/25 (20)
Severe	21/24 (88)	20/25 (80)
Infiltration		
Moderate	5/24 (21)	2/25 (8)
Severe	19/24 (79)	23/25 (92)
Scaling/hyperkeratosis		
Moderate	6/20 (30)	13/23 (57)
Severe	14/20 (70)	10/23 (43)

Efficacy

Both acitretin and hydroxychloroquine produced marked improvement of LE. Erythema responded (complete clearing or marked improvement) to acitretin treatment in 10/24 patients (42%) and to hydroxychloroquine treatment in 17/25 patients (68%). The response rates for infiltration were 15/24 patients (63%) and 17/25 patients (68%), and for scaling/hyperkeratosis 12/20 patients (60%) and 15/23 patients (65%), respectively (Fig. 1). However, complete clearing of LE was more frequent in the hydroxychloroquine group (Fig. 2). In detail, erythema, infiltration and scaling/hyperkeratosis on the face showed slightly higher rates of clearing for each parameter in the hydroxychloroquine group. With regard to the improvement of erythema, infiltration and scaling/hyperkeratosis, there were no significant differences in the response rates between both treatments (chi-squared test; $P > 0{\cdot}05$ for each symptom).

LE involvement of body areas other than the face showed similar results (the data are not shown because of the limited number of patients affected).

In the overall assessment of efficacy by the investigators, complete clearing with or without central atrophy, or marked improvement, was observed in 13/28 patients (46%) in the acitretin group and 15/30 patients (50%) in the hydroxychloroquine group (Table 3). Due to the small sample sizes, the response rate in the acitretin group might be 23% lower to 30% higher than in the hydroxychloroquine group (95% confidence interval for the difference in response rates).

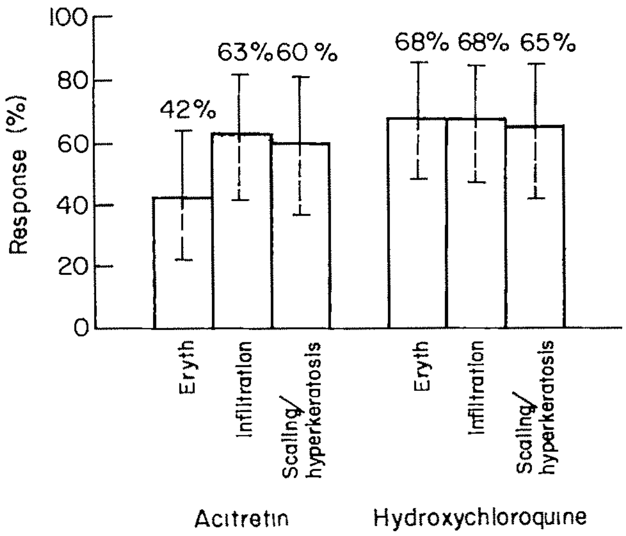

Figure 1. Complete clearing or marked improvement in facial LE lesions—response rates with 95% confidence intervals. Acitretin, $n=24$, hydroxychloroquine, $n=25$.

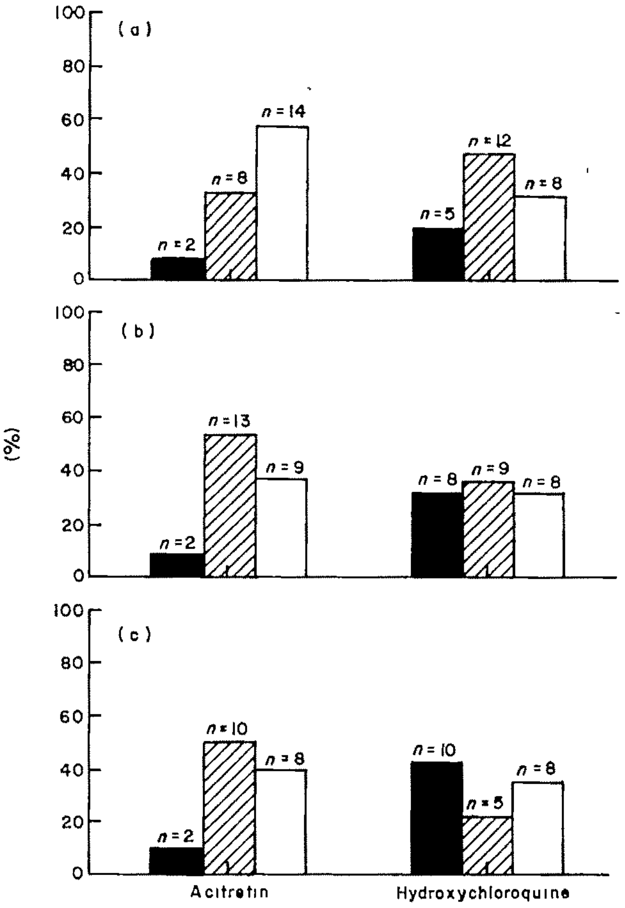

Figure 2. Effects of acitretin and hydroxychloroquine on erythema (a), infiltration (b) and scaling/hyperkeratosis (c). ■, complete clearing; ■, marked improvement; □, no change.

Safety

Twenty-seven of the 28 patients treated with acitretin, (95% confidence interval: 82–100%) and 17 of the 30 patients treated with hydroxychloroquine (95% confidence interval: 37–75%) had at least one clinical adverse

Table 3. Overall assessment of efficacy of acitretin and hydroxychloroquine in cutaneous LE

	Acitretin (%) $n=28$	Hydroxychloroquine (%) $n=30$
Complete clearing	5 (18)	7 (23)
Marked improvement	8 (29)	8 (27)
Slight improvement	6 (21)	6 (20)
No change	3 (11)	9 (30)
Deterioration	6 (21)	0 (0)

Table 4. Adverse events during treatment with acitretin ($n=28$) and hydroxychloroquine ($n=30$) in patients with cutaneous LE

	Acitretin (%) $n=28$	Hydroxychloroquine (%) $n=30$
Skin symptoms		
Dry skin	13 (46)	8 (27)
Scaling/hyperkeratosis	9 (32)	1 (3)
Pruritus/burning sensation	7 (25)	5 (17)
Dermatitis	6 (21)	1 (3)
Others	1 (4)	1 (3)
Mucous membranes		
Dry lips/cheilitis	26 (93)	6 (20)
Dry nose	3 (11)	1 (3)
Conjunctivitis	2 (7)	0 (0)
Dry mouth	0 (0)	2 (7)
Hair/nails		
Alopecia	4 (14)	0 (0)
Paronychia	1 (4)	0 (0)
Laboratory		
Increase in triglycerides	5 (18)	0 (0)
Haematuria	1 (4)	0 (0)
Gastrointestinal tract		
Gastrointestinal symptoms	3 (11)	5 (17)
Others		
General symptoms	10 (36)	6 (20)

event, with mucocutaneous symptoms accounting for most of these. Clinical adverse events are summarized in Table 4. Considering the well-known side-effects of both drugs, no new or unexpected adverse events were noted throughout the study. Additional treatment for adverse events was necessary in 14 patients in the acitretin group, and in 10 patients in the hydroxychloroquine group. Most of these cases were treated with topical application of emollients and sunscreens. Six patients from the acitretin group, in whom adverse events had occurred, responded to a reduction in dosage, and in four other cases medication was discontinued.

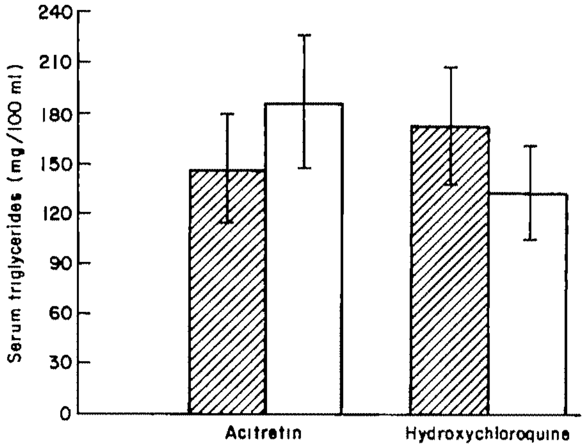

Figure 3. Serum triglycerides before and after treatment with acitretin ($n=28$) and hydroxychloroquine ($n=30$) (mean±2 SEM). ■, before; □, after.

Except for triglycerides, haematology and biochemistry showed no relevant mean changes from baseline values within each treatment group, and no significant differences were seen between both treatment groups. In the acitretin group, triglycerides increased from (mean±SD) $1{\cdot}66 \pm 0{\cdot}80$ mmol/l before treatment to $2{\cdot}10 \pm 1{\cdot}04$ mmol/l after treatment (paired *t*-test, $P=0{\cdot}01$). The corresponding values in the hydroxychloroquine group were $1{\cdot}94 \pm 0{\cdot}98$ and $1{\cdot}50 \pm 0{\cdot}71$ mmol/l (paired *t*-test, $P=0{\cdot}02$; Fig. 3). The comparison between both treatments was statistically significant (non-paired *t*-test, $P=0{\cdot}006$).

Discussion

The aromatic retinoid, etretinate,[13] proved to be a great advance in the treatment of hyperkeratotic and inflammatory dermatoses such as psoriasis. Some similarities in the clinical morphology of cutaneous LE and psoriasis led to the evaluation of retinoid action on LE, which showed benefit in preliminary, uncontrolled studies.[8,9] This, together with the lack of highly effective LE treatment regimens–most available drugs show a response rate in the range of 50% (confirmed in this trial for hydroxychloroquine)–made a further systematic investigation of retinoids in controlled trials imperative. The new aromatic retinoid, acitretin, was chosen for this trial because of its faster elimination kinetics,[1] although some uncertainties concerning the retroconversion of acitretin to etretinate are the subject of ongoing investigations.

The results of this randomized, double-blind, multicentre trial showed comparable therapeutic efficacy in DLE and SCLE for acitretin and hydroxychloroquine, although the sample size sets some limits on the statistical estimation of the effects. Complete clearing and marked improvement were obtained in 46% of the acitretin- and 50% of hydroxychloroquine-treated patients. Detailed analysis of the changes in the target variables showed a somewhat better response rate for hydroxychloroquine. However, the slight asymmetry of the treatment groups, evidenced by the higher number of SCLE patients in the hydroxychloroquine group, may have biased the results in favour of hydroxychloroquine, because SCLE generally responds better to treatment than DLE. An advantage for hydroxychloroquine was shown, however, on analysis of adverse effects, which were more common in the acitretin group and led to premature cessation of treatment in four patients. Overall, the results of this double-blind trial confirm the efficacy of acitretin in the treatment of cutaneous LE suggested by preliminary uncontrolled studies. In addition, the potency of the drug was comparable with that of hydroxychloroquine. Thus, acitretin may represent a valuable addition to the therapeutic arsenal against cutaneous LE. It should be stresed, however, that none of the anti-LE drugs currently available yields satisfactory results when used as monotherapy, as remission rates rarely exceed 50%. Therefore, the combination of low doses of acitretin with hydroxychloroquine will be investigated in future trials, which are being planned at our centres. Such a combination may allow a reduction in dosage of the antimalarial drugs. The interesting observation that hydroxychloroquine leads to a decrease in serum triglyceride levels, which has already been reported by two other groups,[14,15] suggests that this effect may offset the acitretin-induced hypertriglyceridaemia, a side-effect frequently limiting the use of this drug.

Acknowledgments

This study was supported by F.Hoffmann-La Roche & Co. Ltd. We wish to thank Dr Reckers-Czaschka from their Department of Clinical Research for assistance and collaboration.

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British Journal of Dermatology (1992) 127, 519–523.

Coexistent cutaneous T-cell lymphoma and B-cell chronic lymphocytic leukaemia

C.C.HARLAND, S.J.WHITTAKER,* Y.L.NG,* C.A.HOLDEN, E.WONG† AND N.P.SMITH*

Department of Dermatology, St Helier Hospital, Carshalton, U.K.
**St John's Dermatology Centre, St Thomas's Hospital, Lambeth Palace Road, London U.K.*
†St Luke's Hospital, Guildford, U.K.

Accepted for publication 23 June 1992

Summary

Three patients with cutaneous T-cell lymphoma (CTCL) are reported who had concurrent B-cell chronic lymphocytic leukaemia (B-CLL). The separate lineage and clonal nature of the individual lymphoid malignancies were confirmed by gene-rearrangement analysis. The chronology of the illnesses did not support the hypothesis that CTCL predisposes to the development of B-cell proliferative disorders. There was no clear association with immunosuppressive therapy, and HTLV-1 studies were negative in all patients. Consequently, we speculate that a lymphoid stem cell defect, which may lead to the development of either aberrant B- or T-cell clones, may be responsible for such cases of dual lymphoid neoplasia.

The development of immunohistochemical techniques and gene-rearrangement studies has permitted the accurate determination of the lineage and clonality of lymphoproliferative disorders.[1] Although the lineage of lymphomas may be precisely defined by phenotypic studies, cell surface markers can be lost or may be aberrantly expressed in malignancy. In contrast, all lymphocytes undergo unique rearrangements of either their immunoglobulin (Ig) or T-cell receptor (TCR) genes, which can be identified by Southern blot hybridization studies of tissue DNA. Exclusive rearrangements of Ig or TCR genes have been accepted as faithful markers of B- or T-cell lineage, respectively, and indicate the presence of a lymphoid clone.

We report three patients with coexistent CTCL and B-CLL, where separate clonal rearrangements were present in skin and blood, and discuss possible explanations.

Methods

Clinical and laboratory data was collated from patients with coexisting CTCL and CLL whose skin and peripheral blood had been investigated with immunocytochemistry and/or genotypic studies at St John's Dermatology Centre, St Thomas's Hospital, and St Helier Hospital, London. The immunocytochemical and genotypic studies employed in this study have been previously described,[2] but are summarized below.

Immunocytochemistry

Immunohistochemical studies were performed on peripheral blood mononuclear cell cytospin preparations, and either formalin fixed or cryopreserved skin biopsies. Monoclonal antibodies were used in a conventional two-step immunoperoxidase technique. These included T-cell markers, CD3, CD4 and CD8 (DAKO), CD45RO (UCHL1) and CD19, a pan B-cell marker.

Genotypic studies

DNA was extracted from tissue biopsies and from peripheral blood mononuclear cell preparations using a phenol/chloroform extraction and digestion with a urea/SDS lysis solution. DNA samples were digested separately with restriction enzymes EcoR1, Hind III and Bam H1, subjected to agarose gel electrophoresis and Southern blotted on to nylon membranes. Filters were hybridized under high-stringency conditions with a cDNA probe for the constant region of the βTCR chain (Cβ) and a genomic probe for the heavy-chain joining region (JH) of the Ig gene, and then subjected to autoradiography.

Correspondence: Dr C.C.Harland, Department of Dermatology, St Helier Hospital, Wrythe Lane, Carshalton SM5 1AA, U.K.

Table 1. Summary of immunohistochemical and gene rearrangement studies

	Cutaneous tissue							Peripheral blood mononuclear cells			
Patient	CD3	CD4	CD8	CD45RO	CD19	βTCR*	IgH*	IgGκ*	IgGλ*	βTCR*	IgH*
1	ND	++	++	ND	—	GR/GR	G/R	++	—	G/G	G/R
2	+++	++	+	++	—	GR/GG	G/G	ND	ND	G/G	G/R
3	ND	ND	ND	+++	—	GG/GG	G/G	—	++	G/G	R/R

* Results of TCR and Ig gene analysis: R, rearranged; G, germline. Because of the presence of two homologous chromosomes and the configuration of individual TCR genes, there are four germline Cβ fragments, two germline joining region fragments exist for IgH genes. Therefore, results are expressed as haplotypes. +, ++, +++ represent mild, moderate and marked immunocytochemical staining, ND, not done.

Case reports

There was no family history of lymphoid malignancy in any of the cases, and HTLV-1 serology was negative.

Case 1

An 84-year-old man presented in March 1989 with cutaneous lesions of leucocytoclastic vasculitis affecting the lower legs. Investigations did not identify a cause. He had an 8-year history of stable chronic lymphocytic leukaemia, for which no treatment had been necessary. Following resolution of the vasculitis, erythematous plaques developed on the limbs and the trunk over 12 months. Histological examination of skin showed a band-like lymphoid infiltrate in the reticular dermis, epidermotropism, and lymphocytes with convoluted nuclei. Lymphocytes constituted 60% of the white cell differential count in a bone marrow trephine biopsy. Cell marker studies demonstrated monoclonal IgG kappa-bearing B lymphocytes in the peripheral blood (IgGκ), and T cells (CD4 and 8) within the epidermis. Pan-B marker CD19 was negative in the skin. These findings were in keeping with concurrent B-CLL and mycosis fungoides.

In order to confirm the B- or T-cell lineage and establish the clonality, gene-rearrangement studies were performed. Peripheral blood lymphocytes had rearrangement of Ig heavy-chain genes (IgH) with germline TCR genes, while analysis of cutaneous tissue revealed a TCR gene rearrangement and an additional Ig rearrangement identical to that found in the blood. These results confirmed the presence of coexistent B-CLL and CTCL (Table 1).

Case 2

This 87-year-old man presented in 1989 with a polymorphic eruption consisting of scaly erythematous plaques and ulcerated tumours over the trunk and lower limbs. He was also noted to have generalized lymphadenopathy, but there was no evidence of hepatosplenomegaly. B-CLL had been diagnosed in 1982 and had required no treatment. Basal cell epitheliomata had been treated with local radiotherapy in 1984, 1987 and 1989. Treatment of his skin condition with PUVA and superficial radiotherapy had not been successful. His condition rapidly deteriorated before a bone marrow and lymph node biopsy could be performed and he died from septicaemia.

Skin biopsies revealed ulceration of the epidermis overlying an extensive mononuclear cell infiltrate involving the subcutaneous tissue. The infiltrate was composed of atypical cells with hyperchromatic, convoluted nuclei and scattered mitoses. Immunocytochemistry confirmed that these atypical cells were $CD3^+$, $CD4^+$ T cells. Analysis of the peripheral blood mononuclear cell cytospin preparation revealed an increased population of B lymphocytes ($CD19^+$) and reduced levels of T cells, with a normal CD4/CD8 ratio. Analysis of Ig and TCR genes revealed a discrete rearrangement of the βTCR gene and germline Ig heavy-chain genes in DNA from the skin; conversely, there was a rearrangement of the Ig heavy-chain gene and germline βTCR gene in peripheral blood DNA (Table 1). These findings indicated the coexistence of a T-cell clone in cutaneous tissue and a B-cell clone in peripheral blood (Fig. 1).

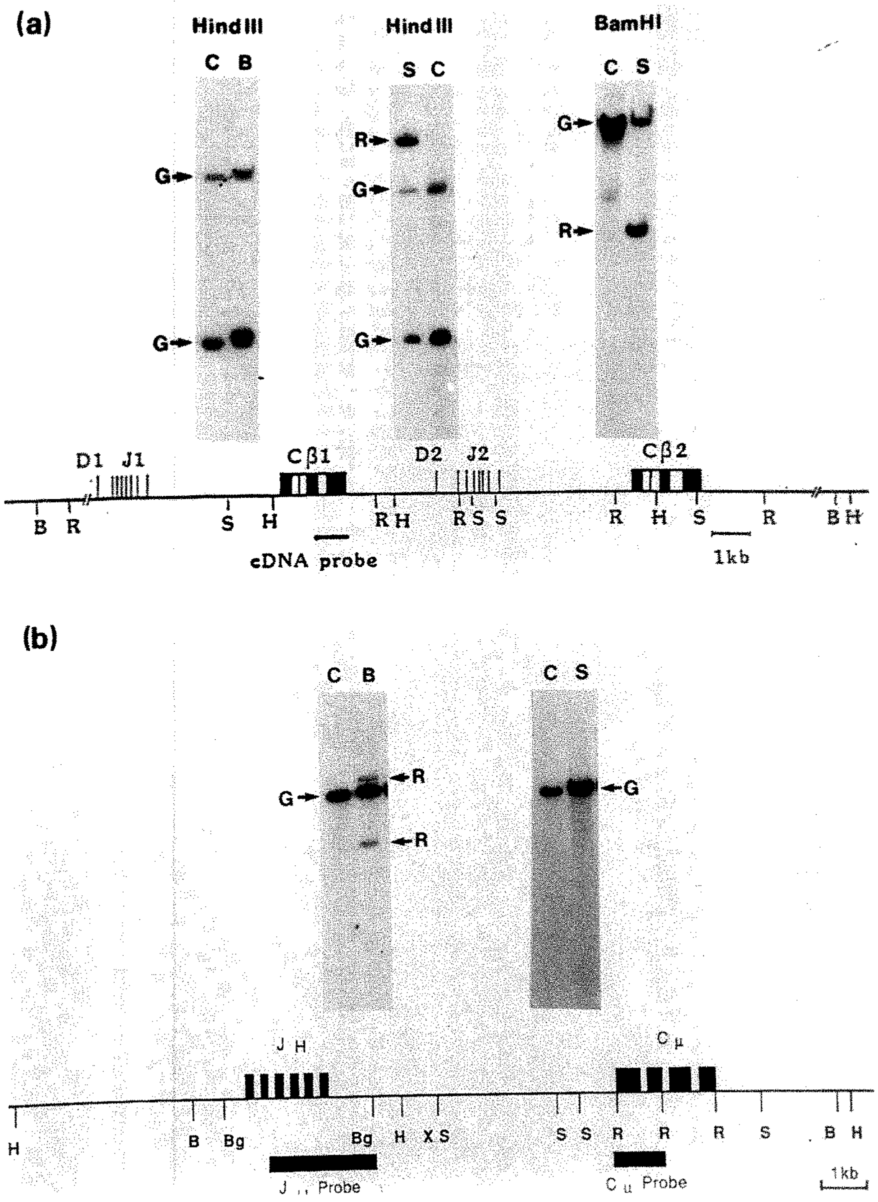

Figure 1. Southern blot analysis of DNA from skin biopsy (S) and peripheral blood mononuclear cells (B) of Case 2, and from peripheral blood mononuclear cells of a healthy individual (C). (a) Digested DNA samples hybridized with a cDNA probe for $C\beta 1$ (as shown on the restriction enzyme map) reveals the presence of a single discrete rearranged band in cutaneous DNA after digestion with Hind III (9·6 kb) and Bam H1 (11·2 kb). These findings are consistent with a rearrangement of $D\beta 2/J\beta 2$ on one allele. In contrast no rearrangements are present in DNA from peripheral blood or control DNA. (b) DNA samples digested with Hind III are hybridized with a genomic probe for the joining region (JH) of the immunoglobulin heavy-chain gene (as indicated on the restriction enzyme map) reveals two discrete rearranged bands in peripheral blood DNA (18·8 and 9·0 kb). This result is consistent with a rearrangement of the immunoglobulin heavy-chain gene on both alleles. In contrast, no rearrangements are detected in DNA from cutaneous tissue or control.
R, rearranged; G, germline band.

Case 3

This 69-year-old woman first presented with B-CLL in 1982 characterized by night sweats, generalized lymphadenopathy, peripheral blood lymphocytosis, and bone marrow involvement. She was initially treated with prednisolone and chlorambucil. Later in the same year she was noted to have an itchy erythematous eruption which fluctuated in severity, and did not respond to treatment with photochemotherapy, ACTH or deoxycoformycin. In 1989 examination revealed extensive, infiltrated, erythematous plaques over the trunk, and scaly erythema over the buttocks and lower limbs. There was axillary lymphadenopathy but no evidence of hepatosplenomegaly.

Skin biopsies revealed a heavy dermal infiltrate composed of large atypical mononuclear cells with convoluted nuclei and a degree of epidermotropism, consistent with CTCL. Immunocytochemistry confirmed that the majority of the infiltrate was composed of T cells (CD45RO^{+}). Analysis of peripheral blood cytospins revealed increased proportions of both CD19^{+} B cells and CD3^{+}, CD4^{+} T cells, which showed morphological features of Sézary cells, with characteristic cerebriform nuclei. However, analysis of DNA from peripheral blood mononuclear cells demonstrated a rearrangement of the Ig heavy-chain gene and a germline βTCR gene configuration, thus indicating the presence of a B-cell clone. Analysis of DNA from cutaneous tissue revealed a germline configuration for both the βTCR and Ig heavy-chain genes (Table 1).

Discussion

Three cases of concurrent lymphoid malignancies of different lineage are described. Cutaneous T-cell lymphoma was present in all cases and coexisted with B-cell CLL. In Cases 1 and 2 the lineage and clonality of blood and skin disease was verified by immunohistochemical studies and analysis of Ig and TCR genes. In Case 3 the presence of atypical lymphocytes with epidermotropism and the immunohistochemical findings were consistent with CTCL, although a malignant clone was not demonstrated. This apparent discrepancy may be due to the limited sensitivity of genotyping; clonal cells should constitute at least 5% of the total cellular population in order to be detected.[2]

The coexistence of B- and T-cell lymphomas is unusual. However, there are several situations in which both clonal Ig and TCR gene rearrangements can be detected. Biondi *et al.*[3] demonstrated that lymphoproliferative disorders can share TCR and Ig gene rearrangements within a single lymphoid clone. Thus, an acute lymphoblastic leukaemia of B-cell phenotype can have simultaneous rearrangement of Ig heavy chain and of TCR regions.[4] Exceptionally, individual cells may also express both T- and B-cell markers. It has been postulated that this 'aberrant' rearrangement is due to the action of a common recombinase enzyme responsible for rearrangement of both Ig and TCR genes.[5] Combined Ig heavy-chain and TCR gene rearrangements have been detected in patients with CTCL.[2,6] It is likely that these are also a consequence of a common recombinase. The Ig heavy-chain gene, being partially rearranged, is rendered non-productive.[7]

Multiple lymphoid clones, which express different immunocytochemical phenotypes, have been shown within the same tissue. Sklar *et al.*[8] described three cases of B-cell lymphoma in which the tumour was composed of two subpopulations of cells, expressing either kappa or lambda light chains with different Ig gene rearrangements, confirming the presence of two lymphoid clones. Similarly, composite non-Hodgkin's lymphomas are described in which immunophenotypic and genotypic studies indicate the presence of both B- and T-cell clones.[9,10] In addition, oligonal B-cell lymphoid proliferations are well documented in patients with Epstein–Barr virus infection and among iatrogenically immunosuppressed recipients of cardiac transplants.[11]

In this series, different lymphoid clones coexisted within separate tissues. The T-cell malignant clone was demonstrated in the skin, whereas the B-cell clone was present in the peripheral blood. Although there are several reports of CTCL in association with B-cell proliferative disorders, especially plasma cell dyscrasias,[12–18] concomitant CTCL and B-cell CLL (or B-cell lymphoma) is very unusual, and has been reported in only three patients.[19–21]

In nearly all reported cases the clinical onset of CTCL has preceded the development of B-cell malignancy. There are various suggestions for this apparent association. Firstly, there is a low but significantly increased risk of second malignancy in patients with CTCL. This observation is not fully explained,[22] but could be due to a delayed carcinogenic effect of therapy with ionizing radiation and alkylating agents. Secondly, it has been suggested that the monoclonal proliferation of helper/inducer T lymphocytes of CTCL may induce the genesis of a B-cell malignant clone;[12] T-cell derived soluble lymphokines, such as B-cell growth factors and interferon, modulate B-cell differentiation and proliferation.[24] Consequently, CTCL may predispose to the uncontrolled proliferation of plasma cells, perhaps accounting for the development of plasma cell dyscrasias. Thirdly, dual lymphoid neoplasms may be attributed to an underlying viral aetiology. A case of CTCL and B-CLL has been reported in association with human T-cell lymphotrophic virus type 1 (HTLV-1) infection.[20] Lastly, dual malignancies may occur by chance, although lymphoproliferative disorders are more likely to occur in patients with a family history of mycosis fungoides,[25] suggesting that there may be underlying genetic factors.

In this series of CTCL and B-cell CLL there was no relevant family history, and no evidence of paraproteinaemia. The clinical presentation of B-CLL predated that of CTCL by several years in two cases, and two patients did not receive intensive chemotherapy or radiation prior to the development of the second neoplasm. There was also no evidence of HTLV-1 infection, although it is possible that other unidentified viruses are implicated.

The temporal sequence of neoplasms in at least two of the patients would be consistent with the conjecture that a B-CLL predisposes to the development of a malignant T-cell clone. There is only circumstantial evidence to support the role of the B cell in the pathogenesis of a T-cell neoplasm.[26,27] Although the coincidence of CTCL and B-CLL may represent a chance association, an additional possibility is that an inherent or acquired defect of a primitive stem cell results in the development of transformed malignant clones of diverse lineage. Thus, the presentation of a T-cell lymphoma would not necessarily predate that of a B-cell-derived lymphoid malignancy. There is evidence that a lymphocyte committed stem cell exists which has the capacity to give rise to mature B and T cells,[28] possibly resulting in the generation of both B- and T-cell clones in rare instances.[29]

With the advent of gene-rearrangement studies, it is possible to distinguish accurately different clones among neoplastic lymphocytes, and in future such studies should establish the frequency of dual or composite lymphomas.

Acknowledgments

We are grateful to Drs R.Emmerson, W.McAllister and J.Behrens for allowing us to report their patients.

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British Journal of Dermatology (1992) 127, 524–528.

Oral etoposide in the treatment of cutaneous large-cell lymphomas. A preliminary report of four cases

J.U.RIJLAARSDAM, P.C.HUIJGENS,* R.C.BELJAARDS, V.BAKELS AND R.WILLEMZE

*Departments of Dermatology and *Haematology, Free University Hospital, Amsterdam, The Netherlands*

Accepted for publication 19 May 1992

Summary The favourable results of oral etoposide as single-agent therapy in four patients with a cutaneous lymphoma other than mycosis fungoides are reported. In all cases other chemotherapeutic options were limited because of prior chemotherapy or the age of the patients. Therapy with etoposide resulted in an initial complete remission in all patients, and was associated with minimal side-effects.

Etoposide (VP16-213), a derivative of epipodophyllotoxin, is a chemotherapeutic agent which was developed 20 years ago. It is frequently used in first-line or salvage regimens for Hodgkin's disease and non-Hodgkin's lymphomas.[1] Used as a single agent, the drug is moderately effective in both newly diagnosed and relapsing non-Hodgkin's lymphomas.[2,3] For cutaneous lymphomas, etoposide has been used both in combination chemotherapy and as single-agent therapy in mycosis fungoides.[4–6] In mycosis fungoides intravenous single-agent etoposide has been reported to induce temporary remission.[5,6] However, there are no reports of the use of oral etoposide in mycosis fungoides or other cutaneous lymphomas. During the past year oral etoposide has been used in our department as single-agent treatment in several patients with cutaneous large-cell lymphomas. The remarkable results of this treatment, with an initial complete remission (CR) in all patients, prompted us to report these cases.

Case reports

Case 1

A 67-year-old woman was seen in November 1989 with multiple tumours on both lower legs. Histological examination showed a diffuse dermal infiltrate of large cleaved cells with expression of CD20, CD22 and κ light chains, typical of a B-cell non-Hodgkin's lymphoma; centroblastic according to the Kiel classification,[7] large non-cleaved cell type according to the National Cancer Institute working formulation.[8] Staging procedures revealed a small lymphocytic B-cell lymphoma in the bone marrow and peripheral blood. The relationship between the cutaneous and systemic lymphoma was unclear, as gene-rearrangement analysis showed different rearrangement patterns for both lymphomas (data not shown). Treatment with six courses of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) resulted in a complete remission of the cutaneous lymphoma, but did not affect the small lymphocytic B-cell lymphoma (Patient 1, Table 1). In July 1990, 3 months after the last course of treatment, two tumours developed on the left arm and left leg. Because of this rapid relapse of cutaneous lesions after CHOP therapy, second-line chemotherapy was rejected. Treatment with chlorambucil 10 mg daily for 2 months resulted in regression of the tumour on the left leg, but the tumour on the left arm enlarged. The patient was subsequently treated with oral etoposide 100 mg daily for 3 weeks. This resulted in a complete remission of the skin tumours within 3 weeks. Apart from hair-loss and a pancytopenia, probably also related to the prior use of chlorambucil, therapy with etoposide was well tolerated. In June 1991, after a disease-free interval of 8 months, a relapse developed on the left arm. Because of the side-effects produced by etoposide 100 mg daily, we treated this relapse with etoposide 50 mg every other day. This resulted in a complete remission after 5 months.

Case 2

A 67-year-old man was seen in November 1987 with several large tumours on his right arm and right leg. Histological examination showed a non-epidermotropic infiltrate of large pleomorphic cells with an aberrant

Correspondence: Dr J.U.Rijlaarsdam, Department of Dermatology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.

Table 1. Clinical characteristics of patients with a cutaneous large-cell lymphoma treated with oral etoposide

	Patient			
	1	2	3	4
Sex/age	F/67	M/67	F/42	M/88
Skin lesions at presentation	Tumours on both lower legs	Tumours on arm and leg	Tumours on scalp	Tumours on arm and trunk
Systemic disease at presentation	Lymphocytic lymphoma; bone marrow and blood	None	None	None
Diagnosis	CBCL, centroblastic	CTCL, pleomorphic large cell	CD30 (Ki-1)$^+$ lymphoma	CD30 (Ki-1)$^+$ lymphoma
Initial therapy	CHOP	CHOP	Prednisone	Etoposide
Result of initial therapy	CR; relapse after 3 months, skin	Relapse during CHOP	Progression	CR
Etoposide dose	100 mg/day	100 mg every other day	100 mg every other day	50 mg/day
Initial result	CR	CR	CR	CR
Side-effects	Hair loss, pancytopenia	Minimal hair loss, fatigue	Nausea	None
Current status	A^0, 30 months*	A^+, 54 months	A^0, 18 months†	A^0, 11 months

CBCL, cutaneous B-cell lymphoma; CTCL, cutaneous T-cell lymphoma; CR, complete remission.
A^+, alive, with disease; A^0, alive, without disease; *months after diagnosis. †complete remission of CD30$^+$ lymphoma; however, relapse of lymphomatoid papulosis.

helper T-cell phenotype (CD2$^-$, CD3$^+$, CD4$^+$, CD8$^-$). Approximately 25% of the T cells expressed the CD30$^+$ (Ki-1) antigen. The lymphoma was classified as a pleomorphic T large-cell lymphoma.[9] Staging procedures, including full blood count, chest radiography, computed abdominal tomography and bone-marrow biopsy showed no extracutaneous disease. Because of involvement of non-related anatomical sites, the treatment consisted of six courses of CHOP. Although all skin tumours initially regressed completely, a new plaque appeared on the right buttock during chemotherapy. The diagnosis of pleomorphic T-cell lymphoma was confirmed histologically. Additional treatment consisted of photochemotherapy with oral 8-methoxypsoralen and topical mechlorethamine (HN2). Despite this treatment, several cutaneous tumours developed, and these were treated with local radiotherapy. When new, poorly demarcated, subcutaneous tumours developed on the right leg and the left arm in July 1990, treatment with chemotherapy rather than radiotherapy was indicated. Because CHOP therapy had achieved only a partial remission, and there was no systemic involvement, second-line combination chemotherapy was rejected and the patient was treated with oral etoposide 100 mg every other day. This resulted in complete regression of all skin lesions within 6 weeks (Patient 2, Table 1). There were a few side-effects, with hair-loss only in the pubic area, and mild fatigue. When, in November 1990, the dose of etoposide was decreased to 100 mg twice weekly a cutaneous relapse on the left arm, left buttock and right ankle developed. The patient currently has persistent cutaneous disease, and is taking etoposide 100 mg every other day.

Case 3

A 32-year-old woman was first seen in 1980 with recurrent papular lesions characteristic of lymphomatoid papulosis (LyP). The LyP was initially treated with PUVA photochemotherapy and after 1982 with topical nitrogen mustard. In 1986 several large, persistent tumours developed on both legs. Histological examination showed a CD30$^+$ T large-cell lymphoma. Staging procedures revealed no signs of systemic involvement. Treatment with two courses of MOPP resulted in a complete remission, although the LyP lesions relapsed within 2 months.

In November 1990 several ulcerating tumours developed on the scalp. Histological examination showed features similar to those of the concurrent LyP lesions. However, in contrast with the LyP lesions, the ulcerating tumours on the scalp did not show any signs of spontaneous regression. Therefore, a diagnosis of CD30$^+$ T large-cell lymphoma was made.[10] Because of the side-effects during previous MOPP treatment in 1986, the patient refused further systemic polychemotherapy. In view of further progression of the tumours during treatment with prednisone 30 mg daily, in March 1991 therapy with oral etoposide 100 mg every other day was commenced. This resulted in complete regression of the ulcerating tumours and rapid clearance of the LyP lesions within 2 months (Patient 3, Table 1). Apart from mild nausea, the patient did not experience any side-effects. She is currently in complete remission, 8 months after discontinuation of etoposide. However, the LyP lesions relapsed 2 months after cessation of treatment.

Case 4

An 88-year-old man presented in June 1991 with multiple firm nodules and tumours on the trunk and upper arm, which had developed over 2 months, and showed no signs of spontaneous regression (Fig. 1). Histology of a tumour on the trunk showed a diffuse, non-epidermotropic infiltrate with cohesive sheets of large anaplastic CD30$^+$ T cells (Fig. 2). Extensive staging procedures revealed no systemic involvement. These findings were consistent with a diagnosis of primary cutaneous CD30$^+$ large-cell lymphoma.[10] Because of the extent of the skin lesions, chemotherapy was considered to be the treatment of choice. However, in view of the age of the patient and the encouraging response in the other cases reported in this paper, it was decided to treat the patient with oral etoposide 50 mg daily, 15 days/month for 4 months (Patient 4, Table 1). After 3 months the skin lesions had cleared completely, leaving slightly pigmented scars. The patient did not experience any side-effects from the treatment. At present he is in complete remission, 4 months after discontinuation of treatment.

Discussion

The majority of primary cutaneous lymphomas other than mycosis fungoides are large-cell lymphomas of either T- or B-cell origin.[11,12] Despite their phenotypic heterogeneity, the therapeutic approach to these primary cutaneous large-cell lymphomas is similar. In patients presenting with solitary or localized skin lesions,

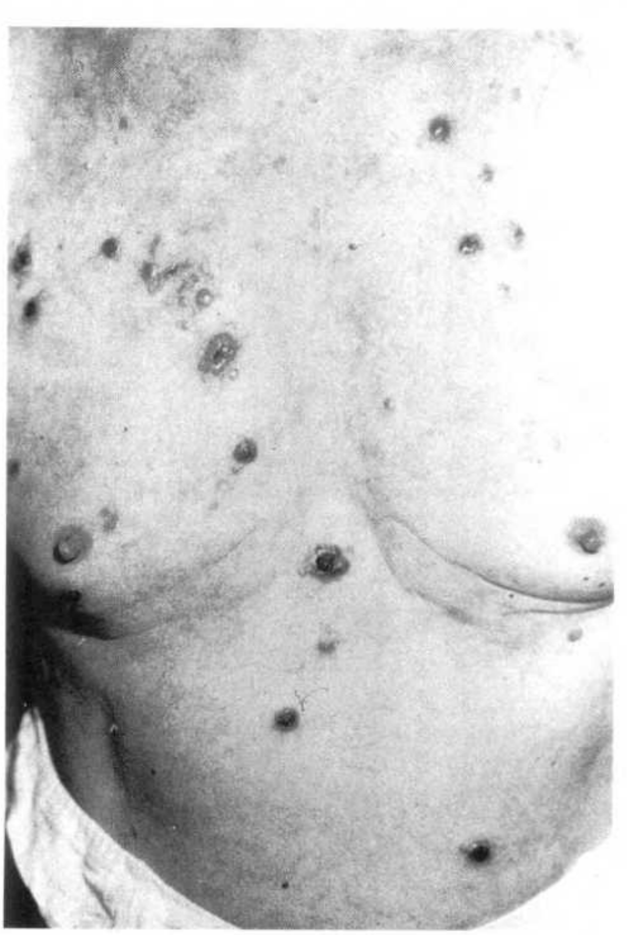

Figure 1. Case 4. Multiple tumours on the chest, with no sign of spontaneous regression.

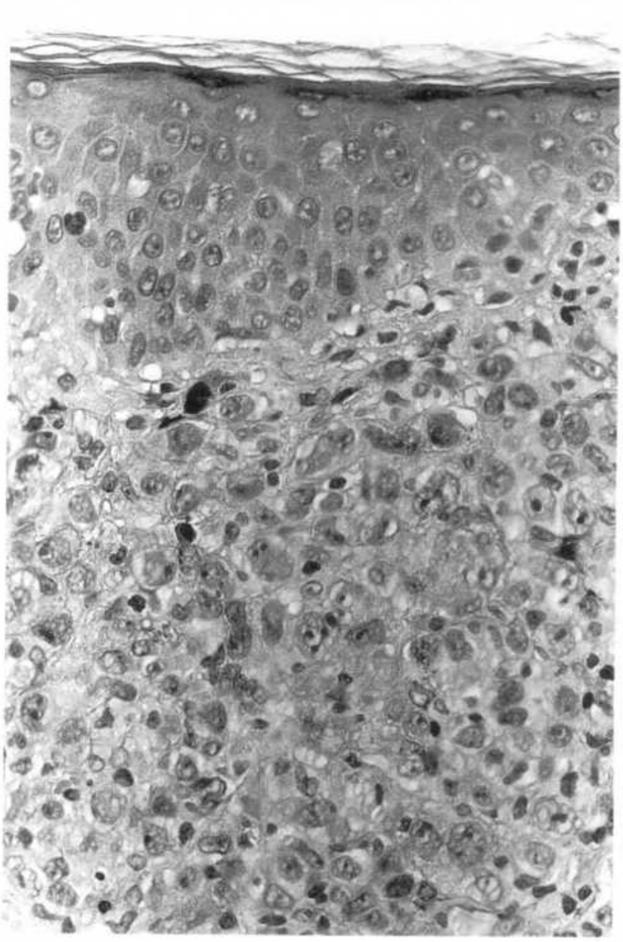

Figure 2. Histology showing a non-epidermotropic infiltrate of large anaplastic T cells with prominent nucleoli and abundant cytoplasm (Case 4) (haematoxylin and eosin ×400).

local radiotherapy is the preferred mode of treatment, resulting in complete remission in the majority of cases. In cases presenting with more widespread skin lesions, polychemotherapy is required. Such patients are generally treated with CHOP or CHOP-like protocols. Irrespective of the type of initial treatment, relapses are seen in a substantial proportion of both B- and T-cell cutaneous large-cell lymphomas. Not uncommonly, these relapses are limited to the skin.[11,14] Solitary or localized cutaneous relapses can again be treated with radiotherapy. However, in the case of a relapse with more generalized skin lesions, radiotherapy is not feasible, and systemic treatment must be considered. The choice of treatment is extremely difficult in patients who experience a relapse during or shortly after initial polychemotherapy, as illustrated by Case 2 in this study. In such patients with skin lesions at multiple sites, and no evidence of extracutaneous disease, who are otherwise in good general health, a curative approach might be favoured and second-line polychemotherapy or, in selected patients, autologous bone-marrow transplantation suggested. However, such an approach may be associated with considerable morbidity and mortality. Moreover, in our experience, it is unlikely that second-line polychemotherapy will produce sustained complete remission. In such patients, a palliative rather than a curative approach may therefore be preferred. Systemic therapy with COP courses or with a combination of prednisone and chlorambucil is often suggested, but is of limited value in patients in whom CHOP-like therapy has failed. Case 2 of the present study was therefore treated, although reluctantly, with PUVA, topical nitrogen mustard and additional radiotherapy for deeply seated tumours. The results of the present study (Cases 1–3) indicate that low doses of oral etoposide as single-agent therapy may be a valuable alternative approach in such cases. In Case 4 etoposide was chosen as initial treatment because of the age of the patient. In all cases complete remission was obtained within 2 months of the start of treatment. In two cases a cutaneous relapse occurred, in one patient (Case 1) 8 months after discontinuation of treatment, and in another (Case 2) shortly after dose reduction during maintenance therapy. Nevertheless, oral etoposide has several advantages over aggressive chemotherapy in the management of cutaneous lymphomas. Because of the availability of an oral preparation, the drug can be prescribed on an outpatient basis. Side-effects, in particular myelosuppression and hair-loss, may occur, but are generally mild and reversible.

The optimum administration schedule of oral etoposide in the treatment of non-Hodgkin's lymphoma is not known, partly because of the variable absorption in individual patients.[14] In several studies oral etoposide 50 mg/m^2 is given daily for 3 weeks.[2,15] If necessary, most of these patients can be treated with a further course starting on day 28. Our cases 2, 3 and 4 show that lower daily doses can also be effective in the management of cutaneous lymphomas. In patients with refractory disease, low-dose maintenance treatment with oral etoposide may be effective.[16] A similar approach has proved effective in the management of refractory systemic non-Hodgkin's lymphoma[2] and small-cell lung cancer.[17]

We admit that the patients in this study represent a small and heterogeneous group of cutaneous lymphomas, that the duration of follow-up is limited, and that the patients were treated with various doses of oral etoposide. Nevertheless, we consider that publication of these preliminary findings is important, because oral etoposide as single-agent therapy proved safe and effective in situations in which alternative methods of treatment would either not be effective, or would be associated with considerable morbidity and mortality.

Further studies are necessary to establish the efficacy and safety of oral etoposide in other cutaneous lymphoproliferative disorders, notably mycosis fungoides and lymphomatoid papulosis. The rapid clearance of LyP lesions in Case 3 suggests that low doses of oral etoposide may be a safe and effective alternative in selected cases of lymphomatoid papulosis.

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British Journal of Dermatology (1992) 127, 529–533.

Recurrent neutrophilic eccrine hidradenitis

E.F.BERNSTEIN,*§ R.L.SPIELVOGEL† AND D.L.TOPOLSKY‡

*Department of Dermatology, Thomas Jefferson University, Philadelphia, PA, U.S.A.
†Division of Dermatology and ‡Department of Neoplastic Diseases, Hahnemann University School of Medicine, Philadelphia, PA, U.S.A.
§Radiation Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

Accepted for publication 1 July 1992

Summary Neutrophilic eccrine hidradenitis (NEH) is a neutrophilic dermatosis primarily affecting the eccrine glands, and most commonly seen in patients undergoing chemotherapy for treatment of a malignancy. Rapid diagnosis may avert unnecessary changes in therapy to treat conditions which clinically mimic NEH. We describe a patient who developed NEH on three separate occasions provoked by two different chemotherapeutic agents—cytarabine and mitoxantrone. The lesions were morphologically distinct and differed in their anatomical distribution during each episode. The response to intravenous corticosteroids was dramatic, but lesions recurred after their withdrawal. This case illustrates the potential diversity of clinical lesions in a single patient with NEH, and its response to systemically administered corticosteroids.

Neutrophilic eccrine hidradenitis (NEH) is a neutrophilic dermatosis primarily affecting the eccrine glands, and occurring most commonly in patients undergoing chemotherapy for a malignancy. It was first described by Harrist *et al.*[1] in 1982 in a patient receiving a cytarabine-containing chemotherapy regimen to treat acute myelogenous leukaemia (AML). Since then, other cases presumably secondary to cytarabine,[2–9] bleomycin,[3,10,11] mitoxantrone,[8,12] chlorambucil,[7] zidovudine,[13] acetominophen,[14] and infection[15,16] have been reported.

Because NEH is most frequently seen in patients receiving chemotherapy, it must be distinguished from disseminated infection, drug hypersensitivity eruptions, leukaemia cutis or other cutaneous metastases, Sweet's syndrome, erythema multiforme, vasculitis, bullous pyoderma, and pyoderma gangrenosum.[1,2,4,5] Clinical differentiation between NEH and disseminated infection may be especially difficult, as both may be accompanied by fever, and may have similar clinical presentations. Thus, establishing the diagnosis of NEH is critical to avoid unnecessary treatment for presumed infections, or changes in therapy for non-existent drug reactions. NEH may even occur in patients with profound neutropenia.[4]

We describe a patient who presented with three separate episodes of NEH with morphologically distinct lesions on each occasion. The third episode of NEH was associated with a different chemotherapeutic agent, and was persistent, but responded to intravenously administered corticosteroids.

Case report

A 43-year-old woman with acute myelogenous leukaemia (FAB type M4) received chemotherapy with daunorubicin (60 mg/m^2 daily for 3 days), cytarabine (25 mg/m^2 day 1, then 200 mg/m^2 continuous infusion, daily for 5 days), and thioguanine (100 mg/m^2 every 12 h for 5 days). In addition she received vancomycin, imipenem-cilastatin, and gentamycin, as empirical treatment for febrile episodes. Allopurinol and blood and platelet transfusions were also administered. Seven days after first receiving chemotherapy, she developed 2·5–3·0 cm, tender, erythematous plaques on her trunk and limbs. Her palms and soles were spared. Many plaques had haemorrhagic or vesicular centres clinically resembling erythema multiforme (Fig. 1). Her temperature at this time was 38°C. The white blood cell count (WBC) was $0{\cdot}6 \times 10^9$ cells/l with 20% neutrophils and 80% lymphocytes. Platelet count was 26×10^9 cells/l. A biopsy of a typical lesion showed a dense infiltrate of neutrophils which were localized around the dermal eccrine glands and proximal eccrine ducts (Fig. 2). There were no neutrophils in the surrounding dermis and epidermis. There was a sparse superficial and deep perivascular predominantly lymphohistiocytic, inflam-

Correspondence: Dr E. F. Bernstein, Thomas Jefferson University Department of Dermatology, 233 South 10th Street, Suite 450, Philadelphia, PA 19107-5541, U.S.A.

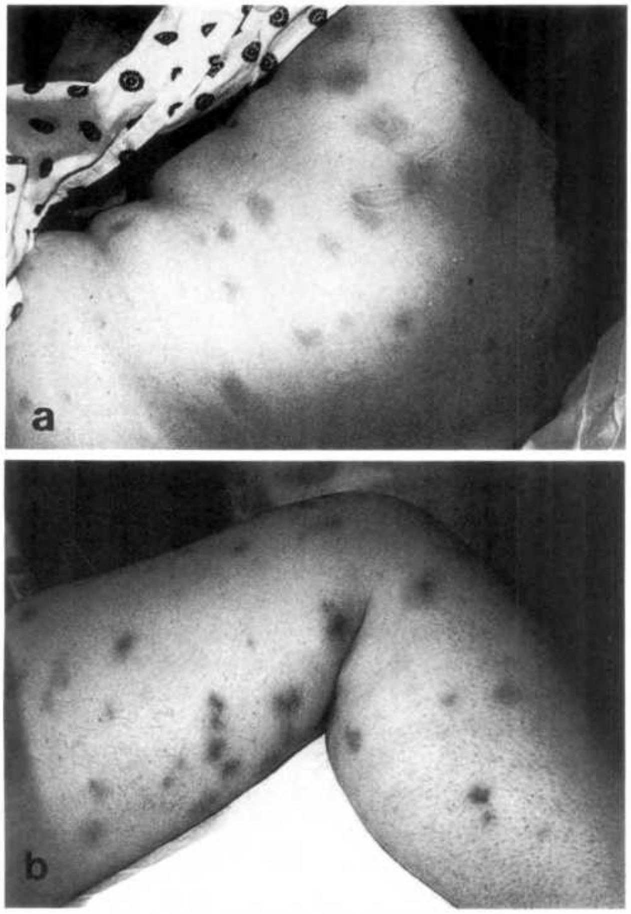

Figure 1. The patient's first episode of NEH following cytarabine therapy. Widespread plaques involving the trunk (a) and limbs (b).

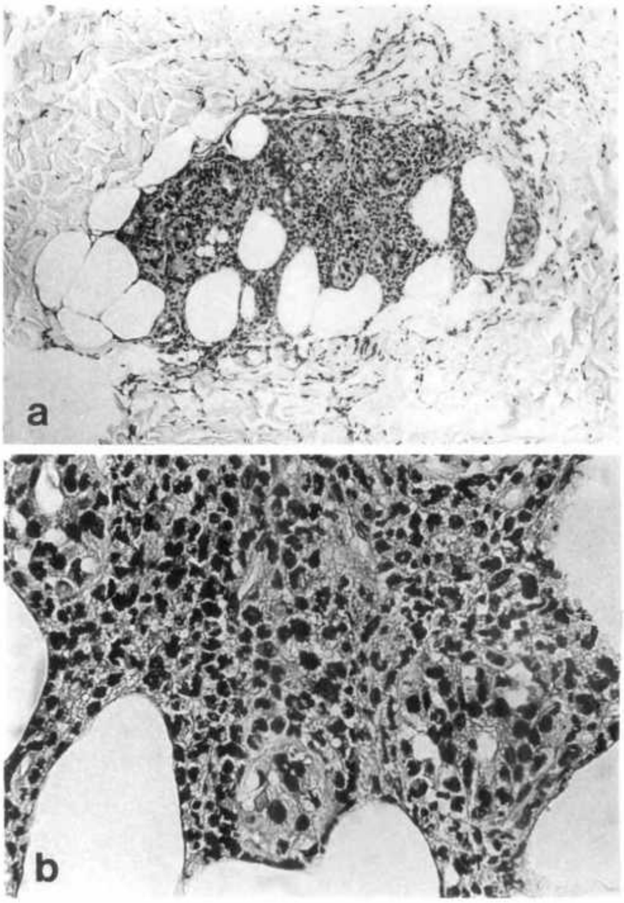

Figure 2. (a) Dense infiltrate discretely localized around the eccrine coils (haematoxylin and eosin × 100). (b) Higher magnification reveals the infiltrate to be composed of neutrophils and demonstrates eccrine coil destruction (haematoxylin and eosin × 400).

matory infiltrate with extravasation of red blood cells. Culture of tissue for micro-organisms was negative. The erythematous plaques became asymptomatic within a few days of their appearance, and resolved slowly over 3–4 weeks.

A bone marrow biopsy performed 12 days after her first course of chemotherapy showed persistence of leukaemic cells. Because of the positive bone marrow biopsy, she was given a second course of chemotherapy with the same agents and doses as originally given. Ten days after the beginning of her second course of chemotherapy several 1·5–2·0 cm, tender cherry-red nodules developed on the upper extremities, sides of the trunk, and neck (Fig. 3). The patient was afebrile, with a WBC of $0{\cdot}6 \times 10^9$ cells/l with 100% lymphocytes, and a platelet count of 26×10^9 cells/l. A skin biopsy revealed findings diagnostic of NEH, and culture of tissue was negative for micro-organisms. The nodules became

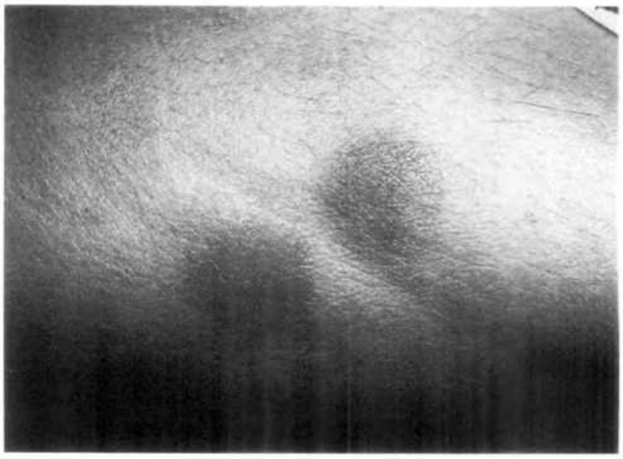

Figure 3. The patient's second episode of NEH following cytarabine therapy. Two characteristic nodules present on the forearm are shown.

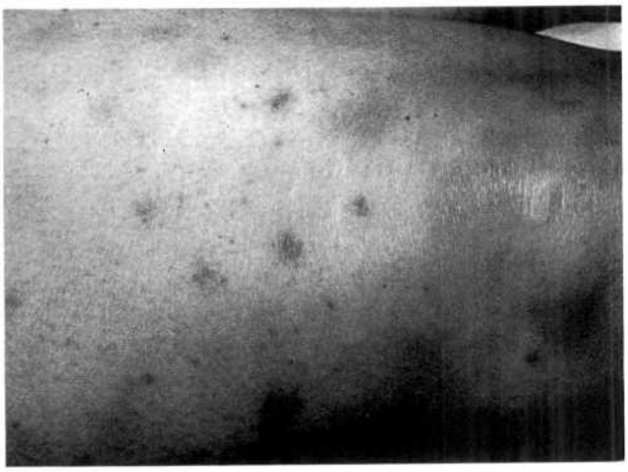

Figure 4. The patient's third episode of NEH following mitoxantrone therapy. Erythematous papules on the thigh.

asymptomatic a few days later, and resolved over the next 3–4 weeks.

Two months after completion of her first cycle of chemotherapy, the patient received a third cycle of consolidation chemotherapy with mitoxantrone (12 mg/m^2 daily for 5 days) and etoposide (100 mg/m^2 daily for 5 days). Early in this course of chemotherapy, vancomycin, imipenem-cilastatin and amphotericin B were started, to treat a persistent fever. Thirty-two days after her first dose of these two agents she began developing numerous 0·4–1·0 cm, very tender, erythematous papules on her limbs (Fig. 4). Her temperature was 37·7°C, WBC 10×10^9 cells/l with 70% lymphocytes and 30% monocytes, and the platelet count was 20×10^9 cells/l. A skin biopsy again revealed NEH. Culture of tissue was negative. However, 2 days after the first appearance of the eruption, the patient developed a temperature of 38·3°C, and many new, exquisitely tender papules. Because of the new onset of fever, metronidizole and tobramycin were added to the patient's antimicrobial regimen of vancomycin, imipenem-cilastatin and amphotericin B. New lesions continued to emerge, and her fever persisted. Methylprednisolone 40 mg/day was administered intravenously. Within 24 h the papules were non-tender and the patient was afebrile. No new papules developed while the patient was receiving methylprednisolone, and the existing papules faded markedly during the following week. She received a total of 3 days of 40 mg/day of methylprednisolone followed by 3 days of 20 mg/day. The patient remained afebrile, but new papules began to develop 2 days after the discontinuation of methylprednisolone, in the identical locations in which they had appeared before steroid administration. Methylprednisolone 20 mg/day was given for 3 days, and the lesions again resolved. However, they began to recur in the same distribution 1 day after steroid therapy was stopped. Antibiotics were discontinued and methylprednisolone was started once again, at a dose of 80 mg/day. The patient was subsequently discharged, and the steroid therapy was tapered over a period of 2 weeks, and eventually stopped. There was no recurrence of lesions or relapse of fever.

Discussion

Our patient's presentation was unique in many ways, and illustrates the heterogeneity of the response pattern seen in NEH. She developed NEH on three separate occasions after two different chemotherapeutic agents. Each episode of NEH was distinct in distribution and morphology. The first two episodes were provoked by cytarabine, and occurred 7 and 10 days after the first dose in a given chemotherapy cycle. Of 13 episodes of NEH reported in the literature where cytarabine was the inciting agent, NEH occurred on average 9·3 days after cytarabine administration.[1–9] In our patient the average time from first administration of cytarabine in a given cycle to occurrence of NEH was similar, averaging 8·5 days. The third episode of NEH in our patient was due to a different agent, and occurred 32 days after initiation of chemotherapy. Mitoxantrone has been implicated in causing NEH 3 weeks after its administration in one report of a patient receiving both cyclophosphamide and mitoxantrone,[12] and 4 days after its administration in another treated with mitoxantrone and etoposide.[8] Our patient also received etoposide and mitoxantrone, again suggesting the latter as the offending agent. Interestingly, our patient developed NEH 32 days after receiving mitoxantrone, but only 7 and 10 days after cytarabine. It appears that the interval between administration of the offending agent and onset of the eruption in an individual may be dependent upon the inciting agent.

In patients in whom the chemotherapeutic agent which initially caused NEH was readministered, four out of eight patients developed a recurrence of the eruption.[1–4,7,10] This was proved by biopsy in only two of the four cases.[1,2] The other two cases were assumed to be recurrent NEH on the basis of the morphology of lesions, the timing of their occurrence, and their location.[4,7] To our knowledge, there is only one other case with more than one recurrence of NEH upon readministration of cytarabine.[7] Our patient demonstrated histologically confirmed recurrences of NEH on three separate occasions, in response to two different chemotherapeutic agents. Although NEH has been attributed to a number of agents in different individuals, the development of NEH in our patient, and one other,[8] receiving both mitoxantrone and cytarabine demonstrates that NEH may be a reaction pattern by an individual, and is not necessarily agent-specific.

Another unique feature demonstrated by this case is the morphological diversity of the lesions during each episode of NEH, despite their marked similarity histologically. Our patient first presented with NEH appearing as numerous erythema multiforme-like lesions in a generalized distribution involving the trunk and limbs. NEH usually presents in a localized distribution involving the limbs or trunk, but not both.[17] Generalized NEH has been described in only three other cases in the literature.[4,8,13] The second occurrence of NEH in our patient consisted of 7–10 cherry-red nodules on her arms, neck, and the sides of the trunk, whereas the third episode consisted of numerous smaller erythematous nodules limited to the limbs. The first two episodes occurred after the same chemotherapeutic agent. Thus, the difference in morphology and distribution was not dependent upon the agent administered.

Our patient was febrile during two of the three episodes of NEH. However, she was undergoing chemotherapy for the treatment of leukaemia and was neutropenic, making infection a likely explanation for her fever. Cultures failed to reveal any organisms, but empirical antimicrobial therapy was started. Eight patients cited in the literature were pyrexial at the time of their presentation with NEH. All were receiving chemotherapy for the treatment of malignancy and were at risk of infection.[1,4,5,7–10] The concomitance of fever with the onset of NEH, the recurrence of fever with each episode of NEH in our patient and two others,[1,7] and the failure to determine a source of infection in our patient, suggest that the fever in these patients was associated with NEH. In none of the eight patients cited in the literature was a source of infection noted.[1,4,5,7–10] One patient had persistence of fever with his NEH lesions, and because the fever was attributed to NEH, antibiotic therapy was stopped. The patient was treated with naproxen 375 mg orally t.i.d. and his fever and skin lesions subsequently resolved. However, spontaneous resolution is the rule with NEH.[10]

The lack of response of NEH to topical treatments is not surprising given the depth of the affected eccrine glands.[11] Our patient's lesions were painful, and were accompanied by fever, and their persistence prompted treatment with intravenous corticosteroids. Both the lesions and the fever resolved within 24 hours of receiving methylprednisolone. However, on two occasions the rash reappeared in the same distribution within 1 or 2 days of discontinuation of corticosteroid therapy. About half of NEH cases are accompanied by fever,[1,4,5,7–10] and tender skin lesions.[1–3,5,7,9,10] Although NEH usually resolves without treatment, skin lesions and fever may persist and therefore warrant treatment. Our patient responded dramatically to treatment, but the decision to administer immunosuppressives to a patient undergoing chemotherapy for treatment of malignancy must be made cautiously.

The exact cause of the neutrophilic accumulation around the eccrine coils in NEH is unknown. Numerous substances have been shown to be chemotactic for neutrophils, including complement component C5a, hydroxyeicosatetranoic acids (HETE), interleukin 1, platelet factor and activating factor, platelet-derived growth factor, and many others.[18] The response of neutrophils in NEH is highly specific. The infiltrate in all three biopsy specimens from our patient was composed almost entirely of neutrophils, despite the absence of any neutrophils on peripheral smear during two of her three episodes of NEH. Katsanis *et al.*[4] reported a paediatric patient who developed NEH on two occasions following treatment for AML. Their patient was neutropenic, with an absolute neutrophil count below 200 × 10^6/l on both occasions during the eruption. Similarly, Gross[8] reported a patient who developed widespread NEH while neutropenic. Thus, the stimulus attracting the neutrophils to attack the eccrine glands and coils in NEH is very potent.

Sweet's syndrome (acute febrile neutrophilic dermatosis) is also characterized by a dense neutrophilic infiltrate, which may be diffuse or perivascular.[19] Clinical lesions in Sweet's syndrome are variable and usually involve the extremities, but may be widespread. As with NEH about half of cases are recurrent, often in similar locations. In addition, fever may be a feature of Sweet's syndrome.[19] Pyoderma gangrenosum is another inflammatory skin disorder where massive neutrophilic inflammation is a characteristic histological feature, and fever may accompany the initial presentation.[20] Both Sweet's syndrome and pyoderma gangrenosum have been described in patients with acute leukaemia,[21–25] and both may respond dramatically to systemic corticosteroid administration.[19,20] The stimulus attracting neutrophils to the sites where they accumulate has not been identified in Sweet's syndrome, pyoderma gangrenosum or NEH. It appears to be very specific, and neutrophil accumulation is limited in many cases to specific anatomical locations in individual patients in all three disorders. The dense neutrophilic accumulation seen in all three conditions may be due to the same stimulus, and identification of the chemotaxin may yield information valuable in their prevention and treatment.

NEH is a neutrophilic dermatosis seen most frequently in patients receiving chemotherapy for the treatment of a malignancy. Because the clinical lesions may resemble disseminated infection, and in view of the few reports of NEH secondary to infection,[15,16] culture of biopsy tissue should be performed. Recurrences are common, and more than one drug may be causative in a single patient. Lesions with a different morphology and distribution may occur with each recurrence. Fever may or may not be a presenting symptom. An absolute neutropenia does not preclude the possibility of NEH. The manifestations of NEH usually resolve within 1–4 weeks without treatment. However, if fever and symptomatic lesions persist, without evidence of infection, systemic corticosteroid therapy may be useful.

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British Journal of Dermatology (1992) 127, 534–537.

Erythropoietic protoporphyria, transfusion therapy and liver disease

D.J.TODD, M.E.CALLENDER, E.E.MAYNE, M.WALSH AND D.BURROWS
Departments of Dermatology, Internal Medicine, Haematology and Pathology, Royal Victoria Hospital, Belfast, U.K.

Accepted for publication 8 June 1992

Summary A 28-year-old man who had suffered from erythropoietic protoporphyria since infancy was referred because of worsening photosensitivity. Conventional therapy with β-carotene, terfenadine and topical sunscreens was ineffective or not tolerated, and he was treated with transfusions of washed packed cells. Unexpectedly, his photosensitivity deteriorated further, his whole blood protoporphyrin levels doubled and he developed abnormal liver function tests. This is the first report of such an adverse response to blood transfusion therapy for erythropoietic protoporphyria and may have been related to subclinical hepatitis or the increased iron load associated with blood transfusion.

Erythropoietic protoporphyria (EPP) is an inherited inborn error of porphyrin metabolism caused by decreased activity of the enzyme ferrochelatase, which catalyses the chelation of iron into protoporphyrin to form haem.[1] It is characterized clinically by photosensitivity commencing in childhood, and biochemically by excessive amounts of red cell protoporphyrin.[2] Protoporphyrin is hepatotoxic, and liver involvement in EPP may be commoner than first reported, although serious hepatic complications are rare.[3,4] If hepatic failure develops it is often rapidly fatal, and regular monitoring of liver function in patients with EPP has been recommended, as there is evidence that early detection of dysfunction and appropriate intervention can prevent or delay serious sequelae.[3,5,6] Blood transfusion therapy has been used in EPP to lower red cell protoporphyrin levels and so improve photosensitivity and liver function.[7–11] Previous reports of patients treated by blood transfusion have described symptomatic and biochemical improvement.

Case report

A 28-year-old taxi driver had suffered from photosensitivity since the age of 4 months, and a diagnosis of EPP was made at the age of 13 years. The diagnosis was delayed because of false negative screening tests for porphyrins.[12] He presented complaining of deteriorating photosensitivity during the previous two summers, and on a bright sunny day could tolerate only 3–5 min exposure before the onset of the typical burning sensation associated with EPP. He could only work at night, and was especially distressed that he was unable to spend time outdoors with his young children. On examination he had characteristic scars over the bridge of his nose and scars, papules and thickening of the skin over the knuckles of both hands.

He was unable to tolerate β-carotene due to unacceptable orange discoloration of the skin, even at low doses. Physical and chemical sunscreens,[13] and terfenadine[14] were ineffective. When the pathogenesis of EPP was explained to him he became convinced that a blood transfusion would be of benefit. Twelve patients with EPP treated by blood transfusion have been reported in the literature, and all improved.[7–11] He understood the risks associated with blood transfusion but because the photosensitivity was severely disrupting his life, and conventional treatment had failed, he subsequently received six units of washed packed cells over a 1-month period. He was not taking any medication during or following the blood transfusion therapy.

The results of investigations pre- and post-transfusion are shown in Table 1. Two weeks after the last transfusion his whole blood protoporphyrin level had doubled, and this was associated with increased photosensitivity and abnormal liver function tests. He could only spend 1–2 min in the sun, and for the first time began to experience burning from sunlight shining through the bathroom window when he was washing in the morning. Appropriate serology for hepatitis A, B and

Correspondence: Dr D.J.Todd, Department of Dermatology, Royal Victoria Hospital, Grosvenor Road, Belfast BT12 6BA, Northern Ireland.

Table 1. Investigations before and after blood transfusion

Investigations	Before transfusion	Post-transfusion			
		2 weeks	6 weeks	2 months	4 months
Whole blood protoporphyrin (normal 70–780 nmol/l)	24,000	49,500	NA	50,258	32,650
Bilirubin (normal 3–18 μmol/l)	9	12	14	16	7
Alkaline phosphatase (normal 35–120 U/l)	125	133	103	105	95
γ-glutamyltransferase (normal 7–46 U/l)	24	49	52	34	32
Aspartate transaminase (normal 10–40 U/l)	30	52	70	53	43
Alanine transaminase (normal 8–35 U/l)	37	118	203	137	84
Serum iron (normal 14–29 μmol/l)	11	33	NA	NA	17
Serum ferritin (normal 100–150 μg/l)	18	290	NA	NA	83

NA, not available.

C, Epstein–Barr virus and cytomegalovirus was negative. Serum ferritin had increased from 18 to 290 μg/l (normal range 100–150 μg/l), but a liver biopsy at 2 months post-transfusion showed the level of liver iron was only 463 μg/g. Mean liver iron in eight normal controls was 700 (±100) μg/g.[15] Over the next few months the whole-blood protoporphyrin level decreased spontaneously, and his liver function improved.

Histology

A skin biopsy from the dorsum of the hand showed thickening of the basement membrane and PAS-positive hyalinized deposits around the dermal blood vessels. Direct immunofluorescence showed deposits of IgA, C1q and C3 components of complement around the thickened walls of the blood vessels, and fine granular deposits of IgM along the dermal–epidermal junction. Indirect immunofluorescence was negative.

A liver biopsy at 2 months post-transfusion showed no evidence of fibrosis or cirrhosis. There was focal mild fatty change of the hepatocytes, with a surrounding sparse lymphocytic infiltrate. Deposits of a golden-brown pigment were noted within the Kupffer cells and hepatocytes. Under polarized light the pigment was bright red in colour, with a central dark Maltese cross typical of protoporphyrin crystals. Intense red fluorescence of hepatocytes was observed on fluorescence microscopy. On electron microscopy there was marked lipofuscin deposition, and lipid droplets within the hepatocytes. Mitochondria varied considerably in size and frequently contained paracrystalline inclusions. Occasional hepatocytes displayed a proliferation of vesicular smooth endoplasmic reticulum.

Discussion

Numerous systemic treatments of EPP are cited in the literature, including antimalarials,[16] inosine,[17] antihistamines,[18] cholestyramine,[19] iron[20,21] and bile acids.[22] Only oral β-carotene has stood the test of time, with over 75% of patients in 23 studies reporting increased tolerance to sunlight when treated with high doses.[23] The only controlled trial of β-carotene in EPP showed no significant improvement over placebo,[24] but this study has been criticised because of the low dose employed.[23] There are five reports of blood transfusion therapy in the literature.[7–11] Photosensitivity improved, and red cell protoporphyrin levels decreased in all 12 patients treated, and the serious hepatic complications in three of the patients resolved. These results, together with our patient's enthusiasm for this form of therapy and his intolerance of β-carotene, prompted us to consider treatment by transfusion. Unfortunately, his photosensitivity deteriorated, and his whole-blood protoporphyrin level doubled to 50,258 nmol/l. This level of protoporphyrin has been associated with hepatic complications.[3]

Of the 12 EPP patients treated by blood transfusion three had serious liver involvement, three had associated hyperviscosity, and six could not tolerate β-carotene or found it ineffective. Six patients were treated by transfusion of packed cells alone, three patients had exchange transfusions, and three patients had plasmapheresis followed by exchange transfusion. The amount transfused and the frequency of transfusions varied considerably, from a minimum 1 unit/month for 5 months to a maximum of 1 unit every 2–7 days repeated 3–10 times. There is one additional report of a patient who received two units of packed cells on two occasions during a 1-month trial of intravenous haematin treatment for his EPP.[25] His red cell protoporphyrin decreased, but it was impossible to assess the part played by the transfusion in the decrease.

Blood transfusion therapy in EPP is thought to act by suppressing haem synthesis and therefore decreasing protoporphyrin production.[7] A dilutional effect would not account for the fall in red cell protoporphyrin level. Why then did our patient not respond to this treatment? It is most unlikely that any of the six units of blood, all from different individuals, contained excess protoporphyrin. One possible explanation is that an episode of subclinical transfusion-associated hepatitis caused release of stored protoporphyrin from the liver. Against this explanation are the facts that washed packed cells were transfused,[10] appropriate serology was negative, and the peak in transaminases followed the elevation in protoporphyrin. An alternative explanation is that the increased iron load associated with blood transfusion caused the deterioration. Oral iron has been reported as causing symptomatic and biochemical deterioration in five patients with EPP,[26,27] and another patient blamed oral iron for increasing her photosensitivity.[2] In contrast, two patients, both with hepatic complications of EPP, improved on treatment with oral iron.[20,21] Why patients should respond to oral iron in such a diverse manner is as yet unexplained. Further studies on the effects of oral iron on red cell protoporphyrin levels in patients with EPP are required.

It is important to realize that the hepatic light, polarization and electron microscopic features can occur in EPP patients whether they have abnormal liver function tests or not,[28] and they cannot be used to predict which patients will develop serious liver disease.[29] Of greater importance is the degree of cholestasis, fibrosis and cirrhosis seen on liver biopsy.[3] These changes are usually associated with abnormal liver function and red cell protoporphyrin levels >20,000 nmol/l. In these circumstances intervention is required to lower the red cell protoporphyrin level and hepatic protoporphyrin load.[5] Cholestyramine, bile acids and blood transfusion have been used for this purpose, and close follow-up is required as hepatic failure can develop very rapidly.[3,22] If this occurs, liver transplantation should be considered, because otherwise there is usually a fatal outcome.[3] This case should make those responsible for the management of patients with EPP aware that intervention with blood transfusion may produce an unexpected deterioration in photosensitivity and liver function.

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British Journal of Dermatology (1992) 127, 538–539.

A case of Sweet's syndrome and myelodysplasia: response to cyclosporin

G.R.SHARPE AND H.M.LEGGAT*

*Departments of Dermatology and *Haematology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K.*

Accepted for publication 18 May 1992

Summary A case of severe Sweet's syndrome associated with myelodysplasia is reported. The skin lesions responded to high doses of systemic steroids, but recurred rapidly on dose reduction. Treatment with a low dose of cyclosporin resulted in sustained clearance of the skin lesions, and was associated with a stable haemoglobin level, without the need for further blood transfusions.

The association between malignancy and Sweet's syndrome is well established, with an incidence of up to 20% in reported cases.[1] The majority of these malignancies are haematological, with acute myeloid leukaemia, lymphomas and myelodysplastic syndromes being the most frequent.[2] We report a case of severe Sweet's syndrome in association with myelodysplasia, which responded well to cyclosporin treatment. Although the development of Sweet's syndrome in myelodysplasia has been reported as heralding progression to acute leukaemia, our patient's haematological state improved with cyclosporin.

Case report

A 50-year-old man presented in 1983 with a macrocytic anaemia (haemoglobin 7·8 g/dl; WBC 4·0 × 10^9/l with a normal differential; platelets 138 × 10^9/l). The bone marrow showed features of a myelodysplastic syndrome. Cytogenetics of the marrow revealed a clonal abnormality with deletion of the long arm of chromosome 7. The patient started folic acid supplements, and required regular transfusions to maintain the haemoglobin above 10 g/dl (Fig. 1). Treatment with oral steroids and stanozolol did not produce any reduction in transfusion requirements. He presented 5 years later at the age of 55 with a 6-week history of a rash which began on the forehead and extended to involve much of the face. The rash consisted of large erythematous plaques with some areas of pustulation, and was initially treated as a recurrence of acne rosacea which had occurred in 1981. The response to topical and systemic antibiotics was limited. Further clinical features suggestive of Sweet's syndrome developed, including pyrexia and episcleritis and the rash extended to involve the scalp, upper trunk and arms. The lesions were erythematous plaques with marked pustule formation, and a number of necrotic bullae. Culture of the pustules was negative and neutrophilia was never present (WBC 2·3–7·1 × 10^9/l, neutrophils < 5·0 × 10^9/l). Skin biopsies confirmed the diagnosis of Sweet's syndrome; histology showed a dense inflammatory infiltrate situated in the dermis and subcutis, which was composed of neutrophils with occasional plasma cells and lymphocytes. Nuclear dust was present, but there was no evidence of vasculitis. The clinical response to prednisolone (40 mg daily) was

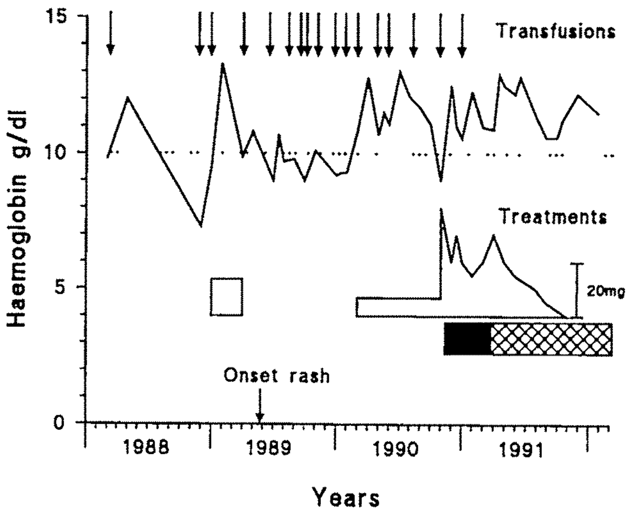

Figure 1. The haemoglobin level over the last 4 years is shown, together with transfusions (↓) and immunosuppressive treatment (□, prednisolone; ■, azathioprine; ▩, cyclosporin).

Correspondence: Dr G.R.Sharpe, Dermatology Department, Royal Liverpool University Hospital, Prescot Street, P.O. Box 147, Liverpool L69 3BX, U.K.

good, although facial lesions took up to 6 weeks to clear. Rapid and severe recurrence of the rash occurred as the daily steroid dose was reduced, and despite adding azathioprine 100 mg daily it was not possible to control the skin lesions with less than 20 mg prednisolone daily. Cyclosporin was substituted for the azathioprine, initially at a dose of 140 mg/day (2 mg/kg), and complete clearance of the skin lesions was achieved within 4 weeks. Prednisolone was withdrawn, and the patient maintained on cyclosporin alone (100 mg daily) with no recurrence of the rash. Since commencing cyclosporin the haemoglobin has remained stable, and further transfusions have not been required.

Discussion

Earlier reports of Sweet's syndrome in association with myelodysplastic syndrome have noted severe skin lesions, as in our patient, with the development of haemorrhagic and bullous lesions.[3,4] A heavy dermal infiltrate of neutrophils, in the absence of peripheral neutrophilia, is also recognized in myelodysplasia.[4,5] Most cases of Sweet's syndrome respond well to systemic steroid therapy, which remains the treatment of choice.[6] Successful treatment with aspirin, indomethacin, colchicine, chlorambucil, dapsone and potassium iodide, has been reported, and spontaneous improvement may also occur, particularly in mild cases. There remain, however, a number of patients who show only partial response to steroids, or in whom the maintenance dose of steroids required to suppress the rash is too high for long-term use. Our patient had severe disease, which did not respond adequately to treatment with prednisolone and azathioprine.

Cyclosporin is established as an effective treatment in a number of inflammatory dermatoses,[7] and was used by Soppi *et al.*[5] in Sweet's syndrome, permitting a small reduction in steroid dose. Cyclosporin is also effective in the treatment of pyoderma gangrenosum,[7,8] which, it has been suggested, may be related to Sweet's syndrome.[3,9] The response to cyclosporin in our patient was dramatic, with complete resolution of all lesions, including areas of ulceration, within 4 weeks. There was no residual scarring and the patient has not developed any further lesions despite complete withdrawal of steroids.

A review of Sweet's syndrome in myelodysplasia suggests that the onset of the skin lesions is frequently associated with the development of acute leukaemia.[5] In our patient, however, there has been no progression of the haematological disease in the 3 years since the development of Sweet's syndrome. In addition, deletions of chromosome 7 are known to indicate a poor prognosis, with the rapid development of acute myeloid leukaemia.[10] After commencing cyclosporin there was, in addition to resolution of the skin lesions, a marked reduction in the number of blood transfusions required (Fig. 1). Whilst taking cyclosporin, currently 100 mg/day, the patient has not required a blood transfusion for 10 months, whereas previously transfusions were required every 4–6 weeks. However, there has not been any significant change in the platelets ($70–130 \times 10^9$/l) or WBC ($3{\cdot}0–5{\cdot}7 \times 10^9$/l) since commencing cyclosporin.

We therefore suggest that cyclosporin is a useful treatment in severe Sweet's syndrome, or in cases only partially responsive to systemic corticosteroids. Moreover, the lack of progression of haematological disease on a low dose of cyclosporin, in a patient with poor prognosis myelodysplastic syndrome is of interest, and the use of this drug in myelodysplastic syndrome merits further evaluation.

Acknowledgments

We are grateful to Drs P.J.Hamilton and J.S.Comaish for allowing us to report this case.

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British Journal of Dermatology (1992) **127**, 540–541.

Neurofibromatosis of the vulva

F.M.LEWIS, M.S.LEWIS-JONES, P.G.TOON* AND T.P.ROLLASON†

*Departments of Dermatology and *Obstetrics and Gynaecology, Wrexham Maelor Hospital, Wrexham, Clwyd, U.K.*
†Department of Pathology, Birmingham Maternity Hospital

Accepted for publication 23 June 1992

Summary Neurofibromatosis can be very variable in its expression. A case of neurofibromatosis presenting with vulval lesions is described.

Case report

A 38-year-old woman presented with a 2-year history of progressive enlargement of the left labium minus, with more recent involvement of the right labium, causing dyspareunia. On examination there was warty hypertrophy of the left labium minus and similar, but less marked, changes on the right (Fig. 1). Histology of a biopsy from the affected area showed the appearances of a benign myxoid neurofibroma. Immunohistochemical staining for S-100 was positive, with numerous small nerves ramifying through the lesion (Fig. 2). Staining for desmin was negative. The patient was examined for other signs of neurofibromatosis. There were two café-au-lait spots only, and no evidence of ocular Lisch nodules, other neurofibromata or flexural freckling. A CT scan of the head was normal. There was no family history of neurofibromatosis, epilepsy or mental retardation.

Localized partial excision of both labia minora was performed, and histological examination confirmed diffuse bilateral neurofibromata.

Discussion

Neurofibromatosis can be variable in its expression, and Riccardi has described eight variants of the disease (Table 1).[1] Ninety per cent of patients develop generalized expression to a greater or lesser extent.

Over 20 cases of vulval neurofibromatosis have been reported, and most of these were in patients with type I neurofibromatosis.[2–4] In fact, vulval involvement in this type of the disease is probably not rare. Schreiber studied 53 women with type I neurofibromatosis and found 10 (18%) with lesions of the vulva.[5]

There have been only two case reports of isolated vulval neurofibromata without generalized neurofibromatosis; both of these were enormous plexiform lesions.[6]

It is important to remember that, occasionally, clitoral enlargement due to neurofibromata can simulate intersex states. This misdiagnosis is easily made if there are accompanying labial tumours which are mistaken for testes.[7]

We have described a patient with late onset neurofibromatosis with lesions apparently localized to the vulva. It is difficult to categorize this patient according to Riccardi's types. The presentation is not truly segmental (type V) in that the lesions are bilateral. However, it has

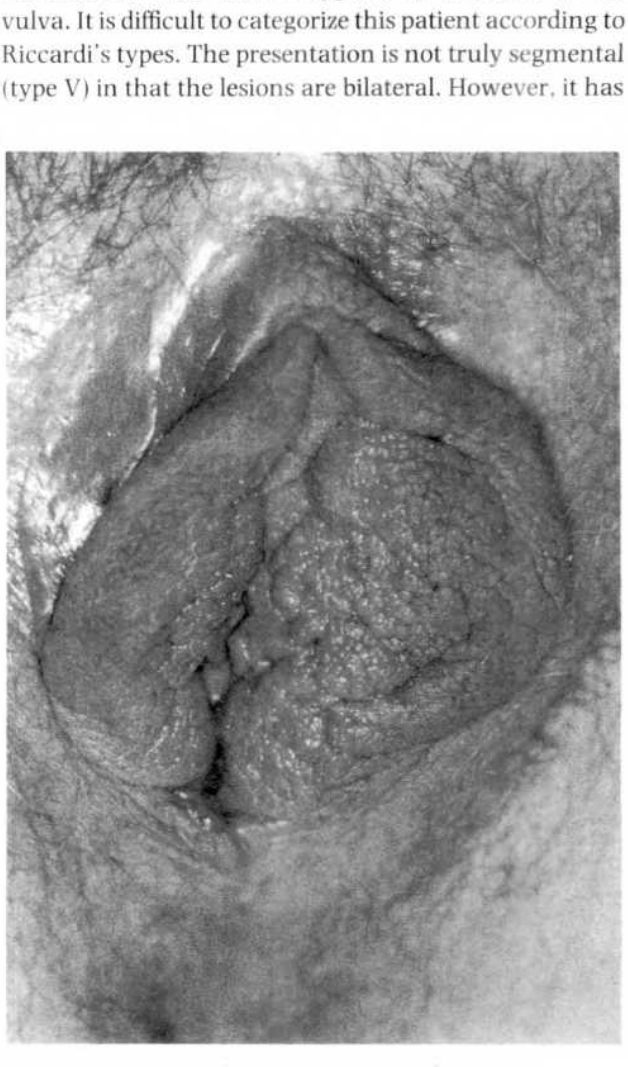

Figure 1. Labia minora showing gross warty enlargement.

Correspondence: Dr F.M.Lewis, Department of Dermatology, Royal Hallamshire Hospital, Sheffield S10 2JF, U.K.

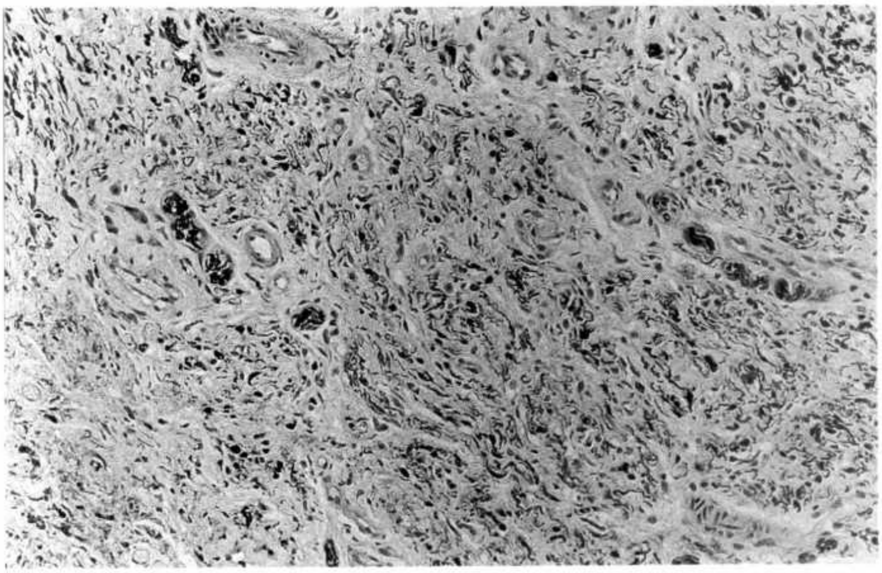

Figure 2. Histology (S-100 stain) showing neural overgrowth and small nerve bundles.

Table 1. Riccardi's classification of neurofibromatosis[1]

Type	Features
I Von Recklinghausen's disease	Multiple café-au-lait spots; Lisch nodules; neurofibromata
II Acoustic	Few café-au-lait spots and neurofibromata; bilateral acoustic tumours
III Mixed	Intermediate between first two types
IV Variant	Café-au-lait spots and neurofibromata; variable family history
V Segmental	Café-au-lait spots or tumours in one unilateral segment
VI Café-au-lait spots	Café-au-lait spots only
VII Late onset	Onset of disease after age 30
VIII Not otherwise specified	Cases not characteristic of any other category

been argued that there is a variant of the segmental type in which a few patients exhibit bilateral dermatomal neurofibromata.[8] In these rare cases, the risk of transmission to the next generation is not fully understood. Our patient has two daughters, both of whom are apparently normal, but have not been examined by us. Unfortunately, the patient has declined further investigation and follow-up.

This patient is an interesting example of the variability of presentation of neurofibromatosis. The diagnosis, although very rare, should be considered in unusual lesions of the vulva.

Acknowledgments

We would like to thank Dr A.Burdge and Dr M.Ridley for their help with this case.

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British Journal of Dermatology (1992) **127**, 542–548.

Correspondence

Clobetasol propionate 0·05% cream in the treatment of vulvar lichen sclerosus: effect on the immunohistological profile

SIR, Lichen sclerosus et atrophicus (LSA) is a chronic dermatosis which predominantly affects the genital areas, and vulvar LSA is a common management problem for gynaecologists and dermatologists. Currently employed treatments, including mild or moderate potency topical steroids and testosterone propionate 2% ointment, usually fail to control disease activity completely, often providing only partial symptomatic relief of vulvar pruritus and dryness.[1–3] Clobetasol propionate 0·05% cream, a very potent fluorinated topical steroid, has recently been reported to improve vulvar LSA both clinically and histologically, without producing significant side-effects.[4] This is in keeping with our experience. Preliminary data from a trial in which 20 cases of vulvar LSA were treated with clobetasol propionate showed that the clinical and histological cure rate was markedly higher than that obtained in a control group treated with topical 2% testosterone.[5] However, longer follow-up is required to confirm this therapeutic effect. Moreover, in some cases, dramatic clinical improvement is not associated with a comparable improvement in histological changes.

The aetiology of LSA is unknown but there is increasing evidence that the skin immune system is involved in disease activity.[6,7] An activated HLA-DR$^+$ T-cell dermal infiltrate, associated with dendritic CD1a$^+$ accessory cells, and an increased number of epidermal CD1a$^+$ Langerhans cells (LCs) is found in untreated vulvar LSA.[7] These findings suggest that LSA should be included in the group of immunomediated dermatoses. It is possible that the epidermal atrophy and dermal fibrosis present in LSA are related to skin immune system activation via lymphocyte production of cytokines capable of modulating keratinocyte proliferation and fibroblast collagen synthesis.[8,9]

We have recently conducted a study in order to establish whether a 3-month period of treatment with clobetasol propionate could modify the above-mentioned immunohistological pattern. An immunohistochemical analysis of vulvar biopsies was performed in 10 women before and after clobetasol treatment (twice daily for 1 month and once daily for 2 months). In seven cases, twice-weekly maintenance therapy was continued for an additional 3 months, followed by a further biopsy (6 months from the start of treatment). A panel of monoclonal antibodies against T cells (anti-CD3, -CD4, -CD8), class II antigens (anti-HLA-DR), Langerhans cells and related dendritic cells (anti-CD1a) was employed, using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Immunohistological evaluation after 3 months treatment showed a dramatic reduction of the dermal lymphoid infiltrate in all cases; only scattered CD3$^+$/CD4$^+$ cells were found in the upper and mid-dermis (Fig. 1). The number of epidermal dendritic CD1a$^+$ cells/100 basal cells appeared reduced (pretreatment $22{\cdot}7 \pm 1{\cdot}9$; after 3 months $6{\cdot}6 \pm 1{\cdot}0$), and HLA-DR staining was confined to dermal vessels and to a

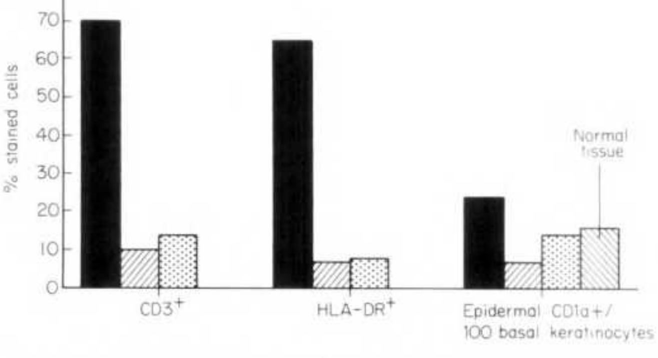

Figure 1. Effect of clobetesol treatment on the immunohistochemical profile. ■, Before treatment; ▨, 3rd month; ⊡, 6th month.

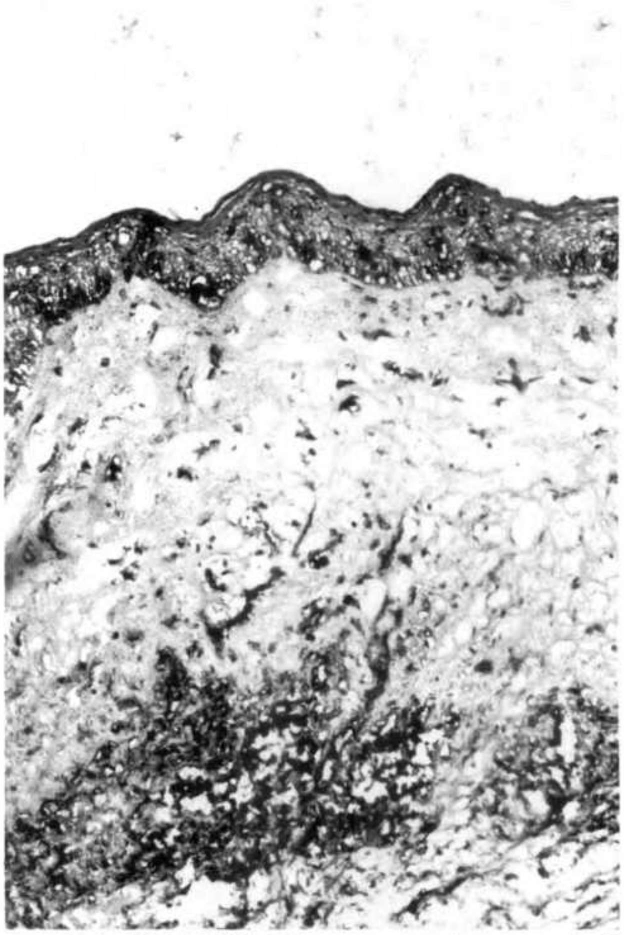

Figure 2. Anti-HLA-DR MAb. Before treatment—the majority of cells in the dermal lymphocytic infiltrate express class II antigens. In the epidermis, dendritic cells are strongly stained.

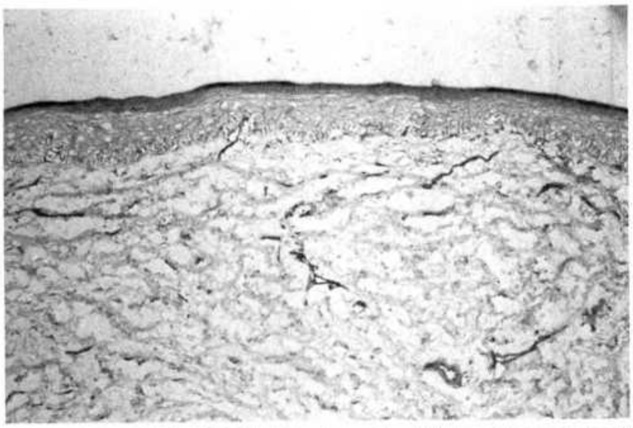

Figure 3. Anti-HLA-DR MAb. After 6 months clobetasol treatment only endothelial staining is found in the dermis.

few epidermal LCs (Figs 2 and 3). In the seven cases who used maintenance treatment, the immunohistological pattern at 6 months remained essentially the same, with regard to numbers of dermal lymphocytes and expression of class II antigens. However, the number of CD1a$^+$ epidermal LCs was increased ($14 \cdot 1 \pm 1 \cdot 9$), almost reaching the level found in normal vulvar epidermis ($16 \cdot 5 \pm 1 \cdot 7$) (four biopsies performed as controls from uninvolved perilesional skin).

In conclusion, treatment with a very potent topical steroid such as clobetasol propionate not only improves the clinical and histological features of vulvar LSA, but also modifies the immunohistochemical parameters of skin immune system activation. These preliminary data suggest that, after a treatment course of 3 months, maintenance therapy is advisable to provide better long-term control of this disease.

Department of Dermatology, P.CARLI
**Department of Obstetrics and Gynaecology,* *A.CATTANEO
University of Florence, B.GIANNOTTI
Italy

Correspondence:
Dr P.Carli,
Clinica Dermatologica II,
via della Pergola 58, 50121,
Florence, Italy

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Potent topical steroid obtained from a Chinese herbalist

SIR, We present a case of a patient obtaining a potent topical steroid without prescription from a Chinese herbalist. A 5-year-old Chinese boy with a 3-year history of atopic eczema was accompanied to a follow-up clinic by his father. His father reported that whilst the 1% hydrocortisone ointment which had been prescribed for his son on his previous attendance had been of little value, there had been a remarkable improvement in his eczema over a 3-month period after obtaining a non-prescription remedy from a Chinese herbalist. However, it was apparent from the packet (Fig. 1) that he had received the potent topical steroid fluocinolone acetonide 0·025%. He had not been warned of the potential side-effects of using this type of preparation and had used it twice a day on his son's face. The eczema had indeed improved, and fortunately there was no evidence of steroid-induced cutaneous damage.

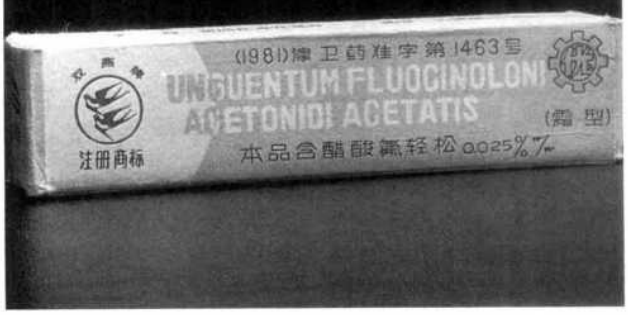

Figure 1.

There are many medicinal compounds used by practitioners of traditional Chinese medicine. In Western countries, although many Chinese and Asian residents will self-medicate or obtain Chinese medicines from a herbalist, Western physicians know little about the various preparations used. Amongst the general population traditional Chinese medicine, as with other 'alternative' therapies, is generally regarded as safer than Western medicine. This view has been questioned recently in relation to topical preparations, as there have been reports of contact hypersensitivity to traditional Chinese medicine.[1,2] This case illustrates the fact that traditional herbal remedies may not always be what they appear. Some Chinese herbalists are illegally supplying prescription-only medicines in this country without adequately explaining to their customers the nature and side-effects of the treatment. In addition, it is curious that in this case a Chinese herbalist should select a potent steroid ointment for the treatment of eczema in preference to a more traditional Chinese remedy.

The Skin Hospital,
Chapel Street,
Manchester
M60 9EP, U.K.

J.O'DRISCOLL
A.D.BURDEN
T.P.KINGSTON

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Acrokeratosis paraneoplastica of Bazex presenting with carpal tunnel syndrome

SIR, We report the association of acrokeratosis paraneoplastica of Bazex and carpal tunnel syndrome.

A 70-year-old man presented to an orthopaedic surgeon in May 1991 with paraesthesia affecting both hands. On examination he was noted to have tenderness over both carpal tunnels, and wrist flexion produced paraesthesia in the median nerve distribution. He was considered to have carpal tunnel syndrome and was referred to a neurologist for further investigation. The tips of the fingers were cold and cyanosed, features which were considered atypical for the carpal tunnel syndrome, and he was then referred for a dermatological opinion. A lump in his neck was also noted.

In September 1990 he had presented with a non-specific eczematous eruption over the trunk and limbs, and changes with a seborrhoeic quality over the scalp and tip of the nose. By November 1990 the tip of his nose had developed an appearance suggestive of cellulitis or rosacea. He was treated with flucloxacillin and subsequently tetracycline, with some improvement. An oesophageal squamous cell carcinoma had been completely excised in 1985. He was taking digoxin, Navidrex® and Oruvail SR®.

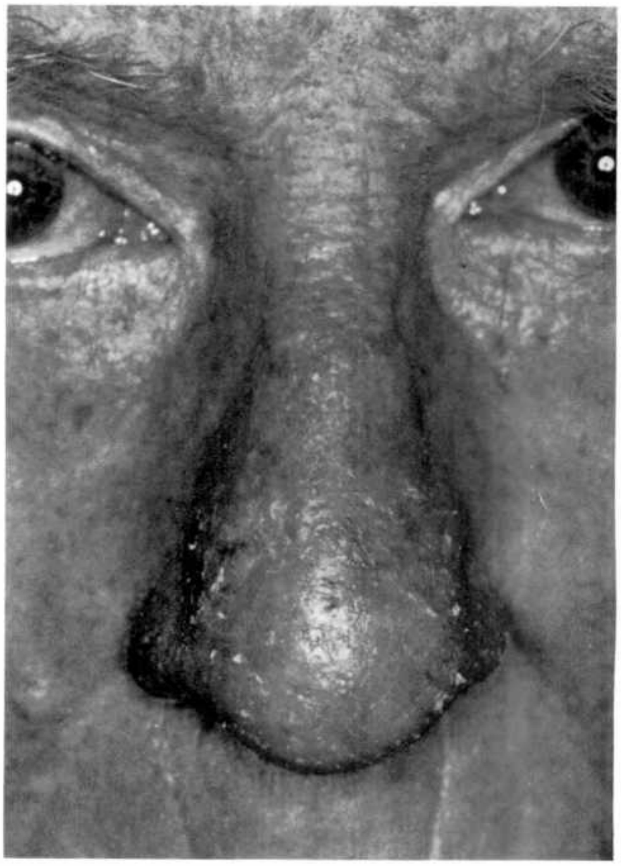

Figure 1. Pityriasiform scaling of the nose.

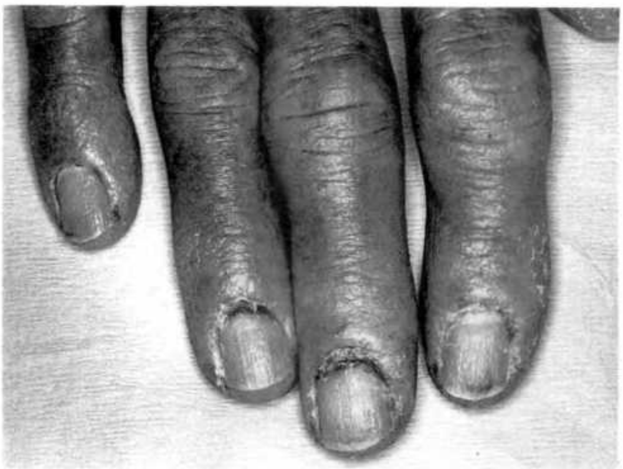

Figure 2. Initial appearance of the fingers.

Examination revealed erythematous scaling of his nose (Fig. 1) and both ears, mildly swollen and cyanotic fingers with distal scaling, swelling of the proximal nail folds, erosion and crusting of the cuticles, scaling of the lateral nail folds, and non-specific longitudinal ridging of the nail plates (Fig. 2). Similar changes were observed on the toes. There was no sensory loss or weakness of his hands. A 2 cm left jugulodigastric lymph node was palpable.

A diagnosis of acrokeratosis paraneoplastica of Bazex was suspected and he was investigated accordingly. Normal findings included: full blood count, liver function tests, blood glucose, creatinine and electrolytes, and chest X-ray. Electromyography demonstrated a mild decrease in motor velocity of the right median nerve (46 m/s) with normal sensory potential amplitude and latencies, and normal sensory and motor conduction along the course of the right ulnar nerve. In the left arm, the motor velocity of the median nerve was at the lower limit of normal. A CT scan of his neck and sinuses confirmed enlargement of the left jugular node, and showed an enlarged right tonsil. Oesophagoscopy revealed a normal oesophagus to the gastro–oesophageal junction, and biopsies were normal. However, biopsies from the right side of the soft palate, right tonsil and right posterior pillar of the fauces all showed squamous cell carcinoma.

Histopathology of the skin showed mild acanthosis, prominent rete ridges, hyperkeratosis, and scattered parakeratotic foci. There were prominent vessels in the papillary dermis and a non-specific infiltrate around the upper dermal vessels (Fig. 3). Immunofluorescence was negative. These features were consistent with acrokeratosis paraneoplastica.

He derived temporary symptomatic relief from topical steroids, and steroid injections in the wrists. He underwent radiotherapy (66 Gy) to the primary tumour site and left upper cervical lymph nodes, and 50 Gy to both sides of the neck.

After 3 weeks of treatment, we observed continuous improvement in his cutaneous signs and neurological symptoms. The abnormalities on the feet were slower to improve than the hands. However, a marked nail-plate dystrophy subsequently became evident.

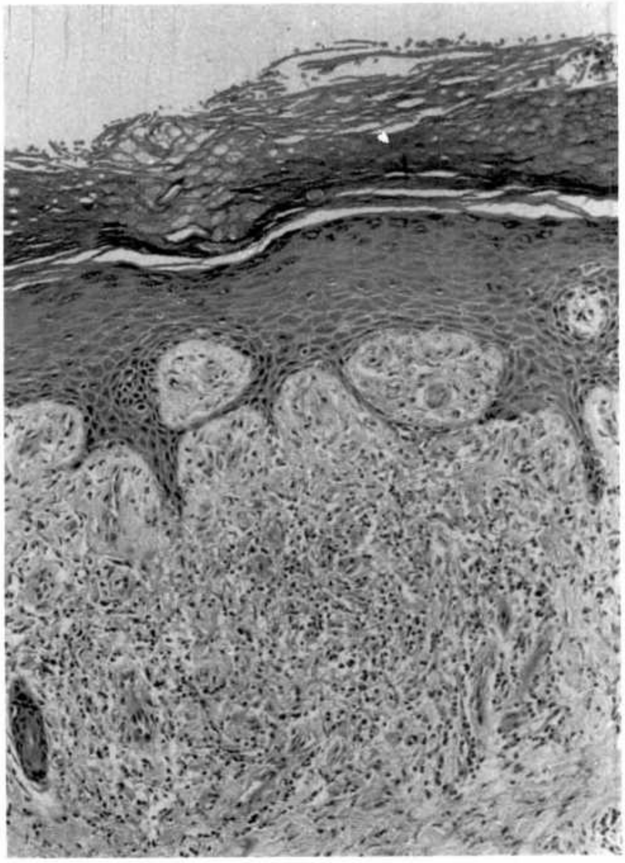

Figure 3. Skin biopsy showing an acanthotic epidermis, hyperkeratosis and parakeratotic foci. Non-specific infiltrate around prominent vessels in the papillary dermis.

There were plans for post-irradiation functional neck dissection, but he developed severe mucositis and marked weight loss, requiring admission for nasogastric feeding. His course was further complicated by aspiration pneumonia, and he died in September 1991. A post-mortem was not performed.

Acrokeratosis paraneoplastica or Bazex syndrome was first described by Bazex *et al.* in 1965[1] as a skin marker of malignancy. Typical features are symmetrical, erythematous, psoriasiform changes on the ears, nose, hands and feet, associated with the presence of a squamous cell carcinoma of the upper respiratory or gastrointestinal tracts. Males over 40 years of age are the classical victims. Although a variety of treatments have produced transient improvement,[1,2] and Wishart[3] reported almost complete clearance of the rash with etretinate, resolution of the eruption usually follows removal of the tumour.

Associated features of Bazex syndrome include acquired ichthyosis, pruritus, vesiculation, sterile paronychia, hyper- and hypopigmentation[1] and bullous lesions,[4] but to our knowledge there have been no previous reports of associated carpal tunnel syndrome. Previously described paraneoplastic peripheral neurological syndromes have involved either axonopathy or demyelination[5] rather than entrapment signs. The concomitant development and resolution of skin signs and carpal tunnel syndrome make it probable, in our view, that the latter was also a paraneoplastic phenomenon.

Department of Dermatology,
Waikato Hospital,
Private Bag,
Hamilton,
New Zealand

B.L.Poskitt
M.B.Duffill

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A nodular cutaneous lymphoproliferative disorder during carbamazepine administration

Sir, Various adverse effects of carbamazepine therapy have been described. About 3% of patients receiving carbamazepine develop skin lesions described as macular, eczematous, or erythrodermic,[1] and rarely bullous[2] or lichenoid reactions.[3] Systemic pseudolymphomatous reactions,[4,5] and more recently localized mycosis fungoides-like lesions,[6] have also been described. We would like to report an unusual nodular lymphoproliferative disorder occurring during carbamazepine treatment.

A 63-year-old diabetic man had been treated with metformin since 1983. In August 1989 he started carbamazepine (400 mg/day) for painful neuropathy of the legs. He was first seen in our department in March 1990 with a 4-month history of skin lesions. Examination revealed numerous erythemato-violaceous, non-scaling nodules, 2–3 cm in diameter, on the scalp (Fig. 1), face, and upper part of the trunk. Two nodules on the back had resolved spontaneously, leaving pigmented scars. Clinical examination was otherwise unremarkable, apart from his neuropathy.

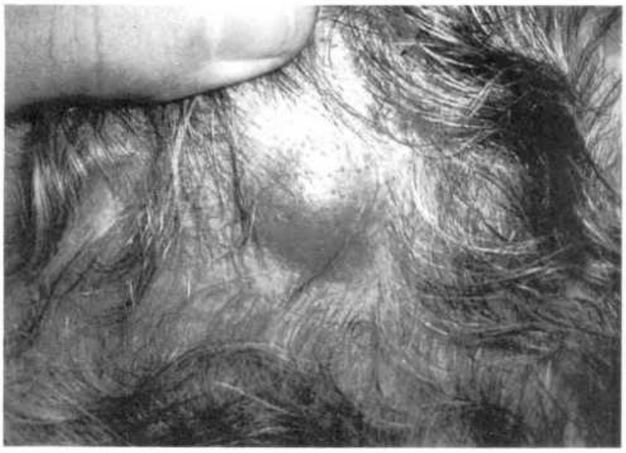

Figure 1. Nodule on the scalp.

The following laboratory investigations were normal or negative: erythrocyte sedimentation rate, full blood count, plasma protein electrophoresis, liver and renal function tests, HTLV1, HIV1, HIV2 and *Borrelia* serology. Chest X-ray and bone marrow were normal. Computerized tomography of the abdomen revealed a 5-cm diameter mesenteric mass of intermediate density, outlined by the small bowel. This extraluminal mass was also seen on small-bowel barium studies. Biopsy of the mass was refused by the patient.

Skin biopsies of a scalp nodule revealed a dense, diffuse dermal infiltrate focally extending into the epidermis (Fig. 2). The infiltrate was principally composed of small lymphocytes, intermixed with some large lymphocytes and epithelioid macrophages. There were equal numbers of small T lymphocytes (UCHL1^{+}), and B lymphocytes (MB2 +). Two types of large cell were observed (Fig. 3). Large B cells (L26^{+}, B 2^{-}) were scattered or in small clusters, and had centroblast-like nuclei. Other large cells, often located in the upper part of the infiltrate, had large angular, cleaved nuclei. These cells were LN2^{+} and S100 protein^{+}, favouring a dendritic origin close to a mononuclear Langerhans cell line. No B-cell monoclonality could be demonstrated.

Carbamazepine was discontinued 6 days after admission. All the nodules resolved within 3 weeks. He remained well 6 months later, and the mesenteric mass had disappeared on a second CT abdominal scan.

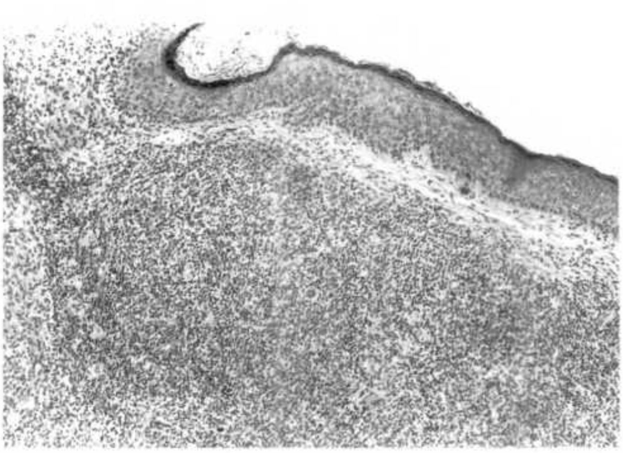

Figure 2. Histology of scalp nodule showing a dense dermal lymphocytic infiltrate (haematoxylin and eosin × 50).

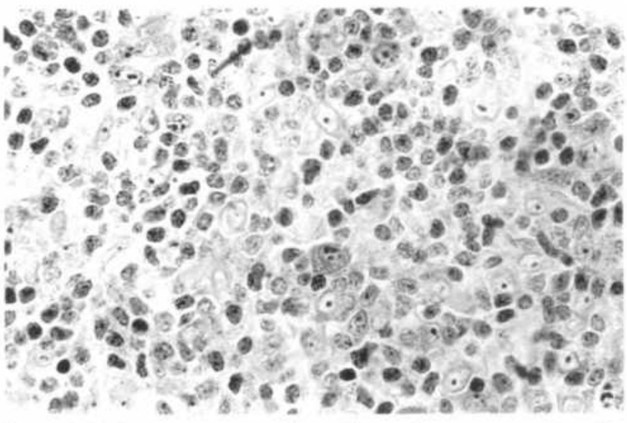

Figure 3. Higher power view of the infiltrate showing centroblast-like large lymphocytes (haematoxylin and eosin × 300).

The clinical, histological and immunohistochemical findings suggested the lesions were benign. Although the patient was not rechallenged with the drug, it appears likely that carbamazepine was responsible for provocation of these lesions. However, coincidental occurrence of a true malignant lymphoma cannot be entirely excluded, as both T- and B-cell lymphomas may show spontaneous regression. In the present case, no B-lymphoid-cell monoclonality was demonstrated, but gene rearrangement to establish a T-cell lymphoid monoclonality was not performed.

Systemic pseudolymphomatous reactions occurring during carbamazepine treatment are well known. Angio-immunoblastic lymphadenopathy is the most frequent histological diagnosis,[4] but cases of carbamazepine-associated lymphadenopathy with histological changes compatible with a diagnosis of non-Hodgkin's lymphoma have also been reported.[5]

Two cases of localized cutaneous pseudolymphoma attributed to carbamazepine have been reported.[6] The clinical presentation was of an infiltrated plaque in one case, and scaling plaques in the other. Histological and immunochemical analyses were suggestive of mycosis fungoides.

Nodular drug-induced pseudolymphoma of the skin is very rare.[7,8] In a reported case treated with cyclosporin, the cutaneous nodules were mainly composed of T lymphocytes.[8] In the present case, histology revealed a polymorphous infiltrate, composed of small T and B lymphocytes and large cells of B-lymphocytic and dendritic origin.

An interesting finding was the demonstration of the mesenteric mass which completely disappeared after withdrawal of the drug. As biopsy of the mesenteric mass was not performed, the relationship with the cutaneous reaction could not be determined.

Departments of Dermatology,
**Pathology, and †Radiology,*
Centre Hospitalier Victor Dupouy,
69 rue du Lt Cnel Prudon,
95107, Argenteuil, France

M.SIGAL-NAHUM
A.PETIT
A.GAULIER*
J.TORRENT†
C.MOURIER
M.KARMOCHKINE

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Aquagenic urticaria masquerading as occupational penicillin allergy

SIR, We wish to report a case of aquagenic urticaria presenting as suspected penicillin allergy.

A 42-year-old man was referred by the occupational health department at the penicillin-processing plant of a well-known pharmaceutical company. Several months after starting work at the plant he began to experience episodes of urticaria. Typically, the attacks developed within 2 h of entering the sterile penicillin powder preparation area in his synthetic, non-porous, protective suiting and mask. Small pruritic weals with a surrounding erythematous flare appeared on the head, neck, arms and upper trunk, but were never associated with angio-oedema. The symptoms lasted for 2–3 h after leaving the plant, and persisted despite relocation to an area with a much lower atmospheric level of penicillin powder. At this stage his continued employment at the plant was in doubt, in view of what appeared to be a severe penicillin hypersensitivity.

However, on close questioning it became clear that these episodes were also occurring to a lesser degree away from the factory. It emerged that the common precipitant was water; this was independent of the temperature of the water and extended to all forms of water exposure, including damp and humid environments. Aguagenic urticaria was confirmed by applying water compresses at 35–36°C to his back for 30 min; small weals appeared at the site of application. Other forms of physical urticaria, in particular cholinergic urticaria, were excluded. In order to explain why episodes occurred whilst working at the plant, when there was no obvious exposure to water, it was postulated that the intensely humid environment created within the non-porous suiting was sufficient to induce urticaria. This was confirmed by wearing the suit on the ward under conditions simulating the factory environment: marked symptoms developed within 1 h.

Although the evidence suggested that the true pathology was aquagenic urticaria, it was important to exclude entirely penicillin as an aetiological agent. Therefore, comprehensive penicillin allergy testing was performed.[1] Intradermal tests to both major and minor antigenic determinants were negative. There were no detectable IgE antibodies to penicillin on radioallergosorbant testing. Patch tests to various dry penicillin powders were negative but, as expected, induced an urticarial response when the powder was reconstituted with water. Finally, oral challenge tests at therapeutic doses produced no reaction.

Urticaria is a common cutaneous manifestation of hypersensitivity to penicillin. In contrast, aquagenic urticaria is a rare form of physical urticaria which was first described in 1964.[2] Typically, it occurs within 30 min of exposure to water, irrespective of temperature, and is usually confined to the upper body. The individual lesions are clinically indistinguishable from cholinergic urticaria, although they are not provoked by exercise or by injection of cholinergic drugs. The pathogenesis is not clear, but histaminergic and cholinergic mechanisms have been implicated.[3] There is also evidence that water may act as a carrier for an epidermal antigen that diffuses into the dermis to cause histamine release from sensitized dermal mast cells.[4]

Patients with aquagenic urticaria respond to antihistamine therapy and the application of inert oily substances before exposure to water.[3] Our patient was started on cetirizine therapy, and as penicillin hypersensitivity had been excluded he was allowed to return to work in an area where protective suiting was not required. He has continued in his employment and his symptoms have considerably improved.

St John's Institute of Dermatology,
St Thomas's Hospital,
Lambeth Palace Road,
London SE1 7EH, U.K.

C.A.HARWOOD
A.KOBZA-BLACK

Acknowledgments

We would like to thank Dr Richard Rycroft, Consultant Dermatologist at the Institute of Dermatology, and Dr Ian Wright, Occupational Health Physician.

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PUVA treatment of psoriasis in Italy

Sir, We read with great interest the recent paper by Farr and Diffey,[1] concerning the location and type of PUVA units, and their operation in the treatment of psoriasis in the U.K. They reported data obtained from 132 (78%) of 170 dermatology departments which were sent a questionnaire in 1989. We also mailed a similar questionnaire to all 135 Italian dermatology departments in 1989 and received 98 (72·5%) replies. A preliminary report of our findings was published in a national Italian dermatological journal.[2] We report herein the full results of our investigation and compare them with the U.K. experience.

In Italy, whole-body UVA devices were not available in 28 (29%) of 98 departments; 14 because of insufficient funding or staff, and four because of doubts expressed about the effectiveness and safety of PUVA; reasons were not provided in 10 replies. These 28 departments were mainly located in southern Italy, and this could be related to the greater potential for sun exposure in the south, which may render PUVA therapy less necessary than in the northern part of the country. The other 70 (71%) centres were equipped with a total of 90 units: 70% of centres used one unit, 24% two and 6% three or more. These data virtually parallel those reported by Farr and Diffey:[1] 134 PUVA units were located in 96 (72%) of 132 dermatology centres in the U.K.; 67% of centres had one UVA device, 28% two, and 5% three. There were 72 conventional UVA fluorescent sources in Italy and 129 in the U.K. However, filtered metal halide lamps were more numerous in Italy (18) than in the U.K. (5). Data about the type of UVA sources are relevant, as their biases of wavelengths within the UVA spectrum may influence therapy.[3] The location of the units was also different. In Italy all but one of the PUVA units were located within dermatology departments, whereas in the U.K. 30% of PUVA treatments were carried out in physiotherapy departments.

In the U.K. there was great variation in the methodology of PUVA treatment for psoriasis. Treatments were given twice-weekly in 24 centres, three times in 67, and four times in 5. The minimal phototoxic dose (MPD) was determined in only five centres, whereas in the other 91 the range of the starting doses for each skin type was surprisingly wide. A great interdepartmental variation was also shown in Italy, but with different ratios to the U.K.; PUVA was given four times/week in 48 centres and three times/week in 16. Both schedules were used in five centres, and PUVA was never used as a twice-weekly regimen. Bath-PUVA was used in one centre. However, the MPDs were always determined in only 23 centres, including three centres in which patients were treated three times/week. Hence, only 20 centres strictly followed the directions of the European Cooperative Clinical Trial.[4] In the other centres, the starting doses were based upon the skin type but, as in the U.K., were not always the same. Additionally, our questionnaire revealed great differences in two other disputed areas of treatment: (i) the maximal UVA single exposure ranged between 7 and 15 J/cm^2; (ii) maintenance therapy was not performed in 22 centres, and always or often in the others.

In summary, the present study, and that of Diffey and Farr, have found a similar ratio of hospitals where photochemotherapy is available, and a similar mean number of UVA sources. However, differences in the type and location of the UVA sources have been shown. In both countries, there is great variability in the methodology of PUVA therapy in different centres, and even greater variability was revealed when the data obtained in the two investigations were compared. These differences make the interpretation of the results and side-effects in studies of PUVA therapy difficult.

Similar studies in other European countries could contribute towards a goal of establishing uniform, optimum PUVA therapy regimes for the management of psoriasis.

Department of Dermatology,
Brescia University Hospital,
p.le Spedali Civili 1,
I-25125 Brescia, Italy
**Department of Dermatology,*
Bologna University,
Bologna, Italy

P.G.Calzavara-Pinton
M.Rastrelli
A.Carlino
F.De Costanza
C.Varotti*
G.De Panfilis

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British Journal of Dermatology (1992) 127, 549–50.

News and Notices

Contact Dermatitis State-of-the-Art Issues, 18–19 June 1993, Hershey, Pennsylvania, U.S.A.

Contact Dermatitis State-of-the-Art Issues will be held on 18–19 June 1993 at The Hotel Hershey, Hershey, Pennsylvania, USA. The programme will include: Cytokines that Mediate Contact Dermatitis; The Role of the Macrophage in Contact Dermatitis; Keratinocyte Participation in Contact Dermatitis; Immunodermatoxicology of Contact Dermatitis; Quantification of Contact Dermatitis with Instrumentation; Specificity and Sensitivity of Patch Testing; Design Considerations for Patch Testing; Controversies in Contact Dermatitis. For further information contact: Jane Mihelic, Continuing Education, M.S. Hershey Medical Center, P.O. Box 851, Hershey, PA 17033, U.S.A. Telephone (717) 531-7965; Fax (717) 533–6618.

14th Colloquium of the International Society of Dermatopathology. The Interdependence of Dermatosurgery and Dermatopathology, 26 June–1 July 1993, Siena, Italy

The 14th Colloqium of the International Society of Dermatopathology will take place on 26 June–1 July 1993 in Siena, Italy. The programme is as follows:

Saturday, 26 June, opening ceremonies in Siena; Sunday, 27 June, scientific session in Florence; Monday, 28 June, self-assessment course in Dermatopathology in Siena; Tuesday, 29 June until Thursday, 1 July, Symposium in Siena.

An incomparable bonus to this meeting is the running of the Palio in the central square in Siena on 2 July 1993. This horse race, run bi-annually for many centuries, is among the most exciting, colourful, and dramatic events in the world. Tickets are available to those registering for the Symposium.

For further information please contact: Dr Lucio Andreassi, Congress Office, University of Siena, Via S. Viglio 6, 53100 Siena, Italy.

International Summit on Cutaneous Antifungal Therapy, 21–24 October 1993, San Francisco

An International Summit on Cutaneous Antifungal Therapy will be held at the Ritz–Carlton San Francisco Hotel on 21–24 October 1993. For further information please contact: Skin Diseases Research Center, 2074 Abington Road, Cleveland, Ohio 44106-5000, U.S.A. Telephone 216-844-3682; Fax 216-844-8993.

Joint Annual Meeting of the British Association of Dermatologists and the Canadian Dermatology Association, 6–10 July 1993, Oxford

The 73rd Annual Meeting of the British Association of Dermatologists will be a joint meeting with the Canadian Dermatology Association under the joint presidency of Dr William Gerstein (Canada) and Dr Terence Ryan (UK).

The meeting will be held in the Academic Centre at the John Radcliffe Hospital, Oxford from Tuesday, 6th July to Saturday, 10th July 1993.

Abstracts of papers and posters are invited for consideration by a Joint Abstract Selection Committee and those accepted as papers will be allotted 10 minutes for presentation plus time for discussion. Abstracts must be submitted on the official forms, which are available from Miss Michelle Hardwicke, BAD Conference Office, 3 St. Andrew's Place, London, SW1 4LB.

The closing date for receipt of abstracts is Friday, 15 January 1993 but applicants should note that the Scientific Committee will be administered by the Canadian Dermatology Association and extra time, therefore, should be allowed for postage to Canada.

Instead of the usual clinical/CPC session held on Saturday morning, there will be a 'Celebration of Dermatology' to be held in the Churchill Hospital, Oxford. Further details will be published in the autumn edition of the BAD Newsletter.

An Update in Dermatopathology, 22 January 1993, St Thomas' Hospital, London

Topics covered will include: Melanocytic Lesions; Lymphocytic Infiltrates; Keratin Abnormalities in Dermatopathology; Connective Tissue Tumours; Oncogenes in Dermatopathology; Malignant Eccrine Tumours; T-Cell Receptor Gene Rearrangements in Dermatopathology and 'New Entities'. For further information please contact: Dr P.H.McKee, Department of Histopathology, St Thomas' Hospital, London SE1 7EH. Telephone 071-928-9292, ext. 2899. (Maximum no. 50.)

A Skin Blood Flow Workshop, The Marriott Hotel, 10 December 1992, San Francisco, CA

A Skin Blood Flow Workshop will be held in the Marriott Hotel, San Francisco on 10 December 1992, immediately after this year's Annual Meeting of the American Academy of Dermatology. For further information please contact: Professor Howard I.Maibach, Department of Dermatology, University of California San Francisco, Box 0989, San Francisco, CA 94143, U.S.A. or Dr Peter Elsner, Department of Dermatology, University of Zurich, Gloriastr. 31, CH 8091, Zurich, Switzerland.

1993 Dermatopathology Course, St Thomas' Hospital, London, Commencing October

The course consists of three, 10-week terms. Classes will be held every Tuesday evening from 6.00 to 8.00 p.m. Didactic lectures are included, but the course consists predominantly of pre-circulated slides. All topics covered. Ideal for the Diploma in Dermatopathology. For further information please contact: Dr P.H.McKee, Department of Histopathology, St Thomas' Hospital, London SE1 7EH. Telephone 071-928-9292, ext. 2899. (Maximum no. 8.)

XV International Pigment Cell Conference, 26–30 September 1993, Kensington Town Hall, London

The International Pigment Cell Conference will be held on 26–30 September 1993, at Kensington Town Hall, London, U.K.

This meeting will be of interest to scientists and clinicians working on melanoma and melanocyte biology.

There will be sessions on the normal melanocyte, disorders of pigmentation, melanogenesis and the molecular biology of melanosomal components. In addition there will be a full day melanoma symposium.

Further details from Professor Riley, the Division of Molecular Pathology, University College, School of Medicine, Cleveland Street, London W1P 6ND, U.K.

The Artemis Fellowships

The Steering Committee of the Nikolas Symposia are offering one or more Fellowships to individuals pursuing research relevant to understanding the biology or pathogenesis, or improving the treatment of histiocytoses, especially Langerhans cell histiocytosis. The Fellowship comprises the full expenses of attendance at the 5th Nikolas Symposium on LCH to be held at the Astir Palace Hotel, Vougliameni, Greece from 7–11 May 1993, plus an award of $1000 to support the Fellow's research.

The closing date for applications is 30 November 1992.

Further details and the application procedure can be obtained from Dr A.C.Chu, Department of Dermatology, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K. Fax 081-740-3169.

British Journal of Dermatology (1992) **127**, 551–559.

Lymphoproliferative responses to human papillomaviruses in patients with cutaneous warts

F.C.CHARLESON, M.NORVAL, E.C.BENTON* AND J.A.A.HUNTER*

Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh, U.K.

**University of Edinburgh Department of Dermatology, Royal Infirmary of Edinburgh, U.K.*

Accepted for publication 23 June 1992

Summary *In vitro* lymphoproliferative responses of peripheral blood mononuclear cells (PBMC) from patients with cutaneous warts, caused by infection with human papillomavirus type 1 (HPV-1) or type 2 (HPV-2), were assayed during the course of treatment. Purified HPV-1 and HPV-2 were used as antigens, as well as herpes simplex virus (HSV) and concanavalin A (Con A). All patients had normal percentages of subsets within the PBMC population and normal lymphoproliferative responses to Con A, and those with a clinical history of HSV infections had positive lymphoproliferative responses to HSV. Responses to both HPV antigens were poor. Only 10 of 100 assays of PBMC from 26 patients showed a stimulation index greater than 2. Addition of interleukin 2 made little difference in most cases. No correlation of clinical status of warts, i.e. improving, unchanged or resolved, with lymphoproliferation was found. When the PBMC were depleted of plastic-adherent cells and enriched for T cells, some samples which had not shown a lymphoproliferative response to HPV-1 or HPV-2 became positive; this response was abolished when the adherent cells were re-added. Thus it is possible that the adherent cell population from a proportion of patients contains cells which suppress lymphoproliferation, or that an immunoregulatory network is present so that lymphoproliferation does not take place *in vitro* without prior activation and cloning of T cells.

Warts caused by infection with human papillomavirus (HPV) types are common, affecting 10% of the population at any one time. The lesions are predominantly persistent in nature and last for several months before resolving spontaneously. About 35% of skin warts resolve in 6 months but, in some apparently healthy individuals, warts may be extensive, recalcitrant to treatment, and persist for many years.[1] It appears that the immune response generated against HPV infection is important in the control of the extent and duration of warts. Clinically this is apparent in patients who are immunosuppressed by disease or drugs, as they will often develop extensive, disabling and persistent warts, particularly when cell-mediated immunity is depressed, rather than humoral immunity. The following clinical examples illustrate this point: (i) pregnant women may develop florid warts which often regress spontaneously following delivery; (ii) patients immunosuppressed by diseases such as lymphomas or leukaemia, or those infected with human immunodeficiency virus, may develop extensive cutaneous and mucocutaneous warts; (iii) patients with epidermodysplasia verruciformis, an autosomal recessive disorder with an associated defect in cell-mediated immunity, have extensive infection with HPV of unusual types;[2] and, (iv) patients immunosuppressed therapeutically following renal or cardiac allografting frequently develop extensive and persistent warts.[3,4]

Histological evidence also points to a role for the immune response in resolution of viral warts. For example, in regressing plane warts caused by HPV-3, an infiltrate of T cells and mononuclear phagocytes is found adjacent to damaged keratinocytes, indicating classical cell-mediated cytotoxicity.[5] On the other hand, such an inflammatory cellular infiltrate is infrequent in other skin warts which are regressing, and is seldom seen in mucocutaneous warts. Furthermore, in clinical terms, the immune response to HPV does not seem to be very effective or long-lasting, with no obvious protection from subsequent episodes of infection.

Assays to demonstrate specific immune responses against HPV have proved difficult, and have often

Correspondence: Dr M.Norval, Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

yielded inconsistent results, with the patient's previous history of HPV infection being largely unknown. In general, no correlation has been found between antibody titre to HPV and a past or present history of cutaneous warts[6] or cervical lesions.[7] Two studies have used *in vitro* lymphoproliferative responses to HPV to assess T-cell immunity in patients with cutaneous warts. In the first, a small response to semipurified HPV was noted in patients with active warts, and this increased after resolution.[8] In the second, patients with warts of short duration (less than 1 year) showed small responses, although these diminished in patients with warts of longer duration.[9] In addition, some controls who thought they had never had warts were positive. It is difficult, therefore, to correlate accurately HPV history and lymphoproliferation. Similar lymphoproliferation results have been reported in patients with cervical HPV lesions and dysplasia, using a variety of HPV antigen preparations, including several fusion proteins representing early regions of the HPV genome. No correlation was found between the clinical diagnosis of cervical HPV infection and positive responses.[7,10]

In the present study, patients with either simple plantar warts caused by HPV-1 or common hand and plantar mosaic warts caused by HPV-2 were followed during the course of treatment. Peripheral blood mononuclear cells (PBMC) were assayed for their ability to respond to purified HPV *in vitro*. As several patients showed improvement or resolution of their warts during the study, correlations between lymphoproliferative responses and clinical status could be drawn. PBMC suspensions were depleted of antigen-presenting cells by plastic adherence, and enriched for T cells by adsorption on nylon wool columns. The responses of the T cells, depleted of, and reconstituted with plastic adherent cells, to HPV antigens were ascertained.

Methods

Clinical samples

The patients studied were attending the dermatology out-patient department at the Royal Infirmary of Edinburgh for treatment of cutaneous warts, and were otherwise healthy. The type of wart present was determined by clinical examination. Two groups of patients are reported—those with simple plantar warts, caused by HPV-1, and those with common hand warts or mosaic warts, caused by HPV-2. The patients were seen and assessed clinically, usually at monthly intervals, and underwent a variety of treatments for their warts such as freezing with liquid nitrogen, application of salicylic acid, or intralesional injection of bleomycin. Age- and sex-matched healthy volunteers, with no known cutaneous HPV infection at the time of sampling, acted as controls. Approximately 50 ml of venous blood was collected in preservative-free heparin at each visit, and processed immediately.

Antigens

Pooled parings from either simple plantar warts (HPV-1) or plantar mosaic warts (HPV-2) acted as a source of HPV antigen. Purified whole virion samples were prepared by caesium chloride density gradient centrifugation[7] and stored in phosphate-buffered saline (PBS) at $-20°C$. Total protein content was determined by the comparison of virion samples with serial dilutions of bovine serum albumin on silver-stained polyacrylamide gels under reducing conditions. The presence of HPV-1 proteins was confirmed by Western blotting of virion samples using a monoclonal antibody for the L1 protein of HPV-1 (Sera-lab, U.K.), and any HPV-2 samples which contained HPV-1 proteins were discarded.

Herpes simplex virus (HSV) antigen was prepared by infecting Vero cells with a clinical isolate of HSV type 1 for 24–48 h at 37°C; the number of plaque forming units (p.f.u.)/ml was assayed by titration in Vero cells before inactivation with ultraviolet B irradiation of 300 mJ/cm^2 (HSV titre before inactivation was 10^8 p.f.u./ml).[11] The lysate from uninfected Vero cells acted as a negative control.[11]

Concanavalin A (Con A) was obtained from Sigma.

Separation of PBMC

PBMC were separated by centrifugation on 'Lymphopaque' (Nyegaard, Oslo) and washed three times in sterile PBS containing 5 U/ml of heparin before resuspension in assay medium (see below). Plastic–adherent cells were isolated from PBMC by incubation in 'Falcon' tissue-culture flasks (Becton Dickinson, U.K.) for 1 h at 37°C.[11] Non-adherent cells were further purified by incubation on nylon wool (Fenwal, Illinois) for 2 h at 37°C to provide a sample which was enriched for T lymphocytes (T-enriched cells).[12] All cell samples were resuspended at 10^6/ml in assay medium which consisted of RPMI 1640 (Northumbria Biologicals, U.K.) with 15% autologous plasma plus 5 U/ml heparin, 2 mM glutamine, 0·05 mM 2-mercaptoethanol, 100 IU/penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamicin and 2 μg/ml Fungizone®. The cell populations were monitored at each stage by flow cytometry using monoclonal antibodies to various cell surface markers (see below).

Lymphoproliferation assay

Cells were seeded into round-bottomed 96-well 'Cell-cult' plates (Sterilin, U.K.) in 200 μl volumes (2×10^5 cells) in the presence or absence of antigen. Con A was added at 10 $\mu g/ml$ and HSV at 1 p.f.u./cell, with the equivalent amount of uninfected Vero cell lysate as a negative control. Approximately 1 μg of HPV-1 or HPV-2 in 20 μl PBS/ml of cell suspension was used, with 20 $\mu l/ml$ sterile PBS as a negative control. PBMC were assayed in quintuplicate for each antigen, whilst T-enriched cells were assayed in triplicate to conserve cell yields. A reconstituted cell sample consisting of T-enriched cells plus plastic-adherent cells (added to form 10% of the total cell number within the same sample volume) was also assayed in triplicate. Cells were incubated in 5% CO_2 at 37°C for 6 days when cultured with Con A or HSV, or 7 days when cultured with HPV antigens. Tritiated thymidine (0·75 μCi in 10 μl PBS/well) was added for the final 18–24 h of culture. Plates were harvested on to 'Titertek' filter paper (Flow Laboratories) using an 'Automash 2000' cell harvester (Dynatech) and discs were counted for 1 min in a 'Tri-Carb' scintillation counter (Canberra Packard).

Some results were expressed as a stimulation index (SI) where:

$$\mathrm{SI}=\frac{\text{mean count per minute (c.p.m.) in presence of antigen}}{\text{mean c.p.m. in absence of antigen}}.$$

An SI value of 2 or more was taken as positive.

Phenotyping of cell populations

Cells were incubated with various mouse monoclonal antibodies, followed by sheep anti-mouse Ig labelled with fluorescein isothiocyanate (Sigma), according to the method of Neill and Miller,[13] except that the first step was performed at 4°C overnight. The monoclonal antibodies which were used are shown in Table 1. Cells were washed twice, resuspended at 5×10^5 cells/ml in cold PBS, fixed with 1% formaldehyde, and approximately 10^4 cells analysed on a Coulter EPICS 'C' flow cytometer (output 100 mW at 488 nm, flow rate 400–500 cells/s).

Results

Clinical status

The clinical details of the patients and healthy volunteers studied are shown in Table 2. The results for patients with common hand warts and plantar mosaic warts are reported as a single group because both are caused by the same HPV type, and no differences were observed between them. In some cases results have been divided into three groups so that immune responses could be correlated with changes in clinical status. These were: (a) no improvement; (b) improvement (i.e. a decrease in the number of warts or degree of hyperkeratosis) or, (c) complete resolution ('resolved'). Patients were monitored over varying lengths of time according to changes in their clinical status.

Table 1. Monoclonal antibodies used in phenotyping by flow cytometry

Cell surface marker	Target cell	Monoclonal antibody	Source
CD3	T lymphocytes	DAKO-T3	Dakopatts
CD4	T-helper lymphocytes	DAKO-T4	Dakopatts
CD8	T-suppressor/cytotoxic lymphocytes	DAKO-T8	Dakopatts
CD1a	Thymocytes Dendritic cells	DAKO-T6	Dakopatts
MHC class II	Monocytes/macrophages B lymphocytes Activated T lymphocytes	DA6.231	Dr K.Guy[§]
CD57	LGL[*] NK cells[†]; K cells [‡]	Leu 7	Becton Dickinson
CD11b	Monocytes T-suppressor lymphocytes LGL/NK cells	OKM1	Becton Dickinson

[*] LGL, large granular lymphocytes.

[†] NK, natural killer cells.

[‡] K, killer cells.

[§] Kind gift from Dr Keith Guy, Western General Hospital, Edinburgh.

Table 2. Clinical history of study groups

Diagnosis	HPV type	Number in group (male, female)	Mean age (years)	Duration of wart(s) (years)			
				<1	1–5	5–10	>10
Simple plantar	HPV-1	9 (5M, 4F)	27·8 (range 17–46)	2	3	3	1
Common hand warts	HPV-2	11 (9M, 2F)	38·9 (range 22–70)	4	2	4	1
Plantar mosaic	HPV-2	6 (3M, 3F)	39·3 (range 20–62)	0	5	0	1
Healthy controls	—	11 (5M, 6F)	37·9 (range 24–62)	—	—	—	—

Table 3. Phenotype of peripheral blood mononuclear cells in HPV patients and controls

Clinical status	Number of samples	CD3	CD4	CD8	CD1a	MHC Class II	CD57	CD11b
HPV-1 warts								
No improvement	8	45+13	34+12	21+6	1·2+0·4	19+7	13+7	27+10
Improvement	7	53+15	37+6	24+6	3+2	20+6	18+6	32+10
Resolved	3	49+9	42+5	21+2	1+0·4	21+1	11+8	23+0·5
HPV-2 warts								
No improvement	9	48+10	35+11	20+8	1·6+1	29+13	13+8	30+13
Improvement	16	44+8	36+10	21+7	1·9+0·9	26+8	16+8	32+10
Resolved	5	54+12	37+8	25+2	2·8+1·9	16+9	14+8	27+17
Normal controls	9	45+7	33+11	24+7	3+1·9	21+6	16+9	29+9

Values represent the mean % positive+1SD.

Phenotyping of PBMC

Table 3 shows the expression of various cell surface markers on PBMC from patients in the different clinical groups, expressed as a percentage of the total number of cells analysed. There was no significant difference in the PBMC phenotype of any group at a particular clinical stage. The phenotype of individual patients did vary in some cases, but this was observed both for patients who showed clinical progress as well as those who did not, and there was no correlation between changes in phenotype and change in clinical status (data not shown).

Lymphoproliferation assays

The *in vitro* mitogenic response to Con A was consistently high in all PBMC samples. In those patients and control subjects who reported a clinical history of HSV infections (14 of 37), the proliferative response to HSV was high, the stimulation index varying from 2·4 to 26·8 with a mean of 7·4.

The proliferative responses to HPV antigens of PBMC from patients with warts caused by either HPV-1 or HPV-2 are shown in Figures 1 and 2, respectively. In both cases, most patients showed no responses to HPV antigens at any stage of their wart infection, nor did any of the controls. Indeed only 10 samples out of a total of 100 assays showed a stimulation index greater than 2. Patients who showed a positive response to at least one HPV antigen sample had had their warts for at least 1 year; in one case more than 6 years and in another more than 10. The patients who showed no response to HPV antigens had wart infections for less than 1 year to more than 10 years.

Assays were also carried out using the E4 proteins of HPV-1 and HPV-2 expressed as fusion products with β-galactosidase of *E. coli* as antigen.[14] They proved unsuccessful because, in almost all subjects, there was a strong lymphoproliferative response to the β-galacto-

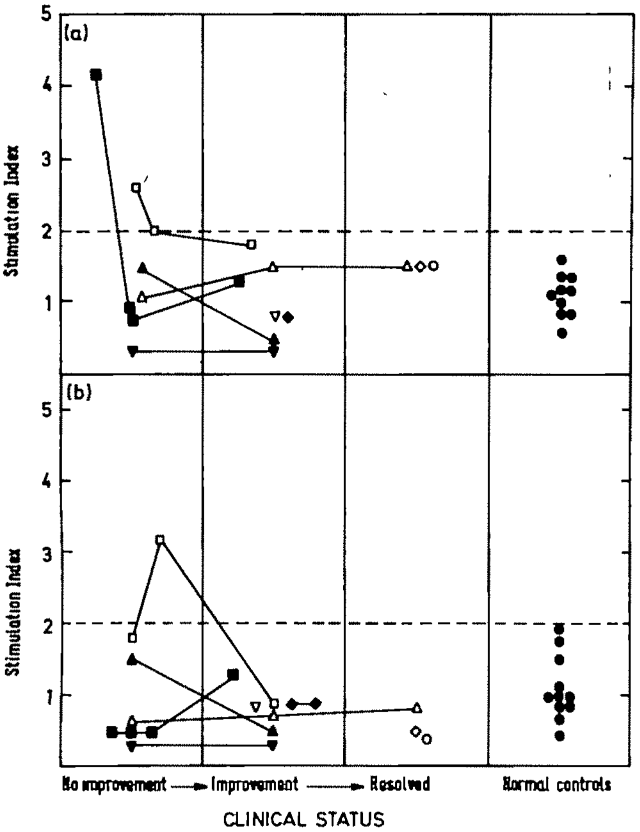

Figure 1. *In vitro* proliferative responses of PBMC from patients with HPV-1 type warts to whole virion samples of (a) HPV-1 and (b) HPV-2 during changes in clinical status of the wart infection. Points joined by a line indicate successive samples from the same patient as the wart infection progresses from no improvement, through improvement, to resolved. In the normal controls' column, points represent samples from individual members of the control group.

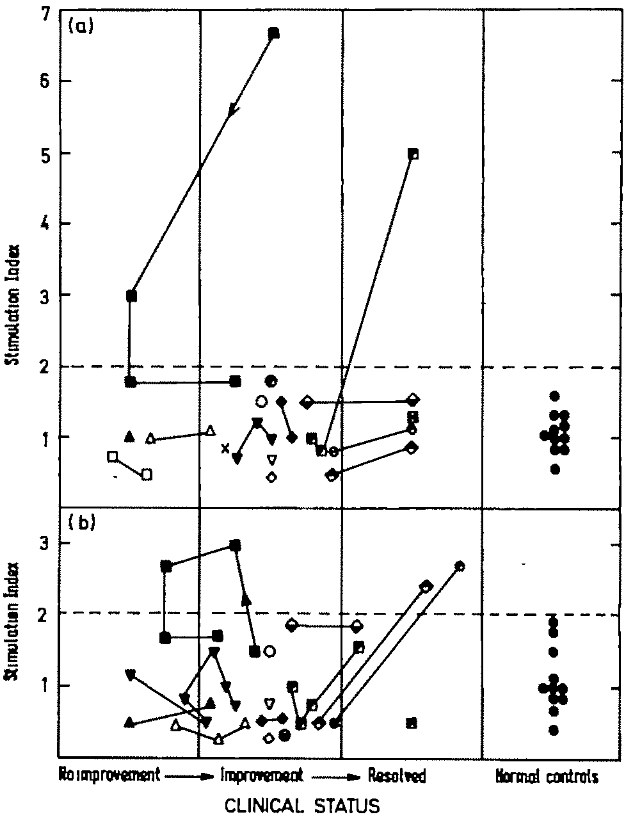

Figure 2. *In vitro* proliferative responses of PBMC from patients with HPV-2 type warts to whole virion samples of (a) HPV-1 and (b) HPV-2 during changes in clinical status of the wart infection. Points joined by a line indicate successive samples from the same patient as the wart infection progresses from no improvement to resolved. The clinical status of one patient changed from improvement, to no improvement, before improving again (■——■).

sidase protein itself which was of the same magnitude as the response to the fusion protein.

In four cases, exogenous interleukin 2 (IL-2) was added to lymphoproliferation cultures to see if this would augment responses to HPV antigens. IL-2 was added on day 4 of culture at 10 U/well in 10 μl sterile PBS. This had no effect on the negative response of a healthy control, nor on that of a patient with warts caused by HPV-2 which were not improving. In another patient with unimproved warts caused by HPV-2, a strong response to HPV-2 virions was seen only after the addition of IL-2, and in a third patient whose HPV-2 type warts were improving, a small positive response to HPV-1 virions became negative after the addition of IL-2.

Studies of separated PBMC populations

PBMC samples were further purified to obtain a population of plastic–adherent cells and a population enriched for T lymphocytes (T-enriched cells). The phenotype of these populations in the different clinical groups is shown in Figure 3. All T-enriched cell samples showed an increase of approximately 20% for CD3-positive T lymphocytes, compared with the whole PBMC populations, with very few CD3-positive cells remaining in the adherent cell samples. Class II MHC-positive and CD11b-positive cells were enriched in the adherent cell samples by between 20 and 40% compared with the whole PBMC samples, whilst cells bearing these markers were depleted in the T-enriched samples, although not to absolute levels. No differences were apparent between the percentages of positive cells in the two patient groups and the control subjects.

Table 4 shows examples of the responses to HPV-2 by whole PBMC populations, and reconstituted populations where adherent cells have been re-added to T-enriched

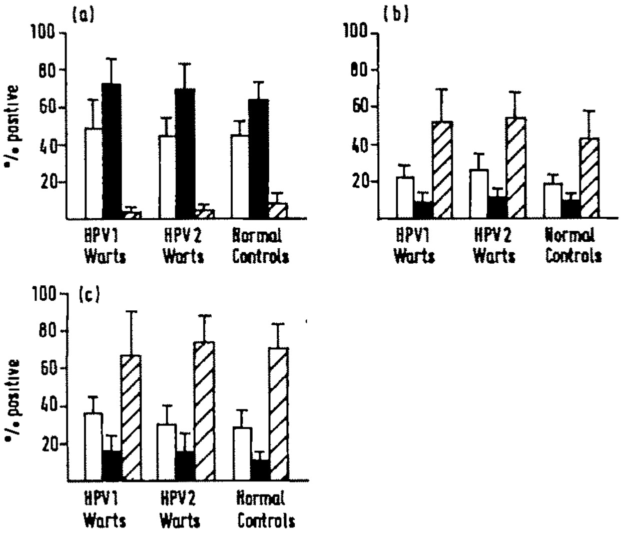

Figure 3. Phenotype of separated PBMC populations by flow cytometry using monoclonal antibodies specific for (a) CD3, (b) class II MHC and (c) CD11b. □, PBMC; ■, T-enriched cells; ▨, plastic-adherent cells.

cells to form 10% of the total cell numbers and sample volume. None of the populations in patient 44A shows a positive response to HPV-2. The PBMC populations in patient 34B show a positive response which is depleted in the T-enriched population and subsequently restored when adherent cells are re-added. Conversely, the PBMC sample in patient 46B shows no response, whereas the T-enriched sample does, although this response is abolished when adherent cells are re-added. These lymphoproliferative patterns of PBMC and T-enriched populations were observed in all clinical groups in response to both HPV-1 and HPV-2 antigens (Table 5). On the other hand, the response to HSV was different. In those patients without a clinical history of HSV infection, the response of all cell populations to HSV antigen was negative. In those patients with a clinical history of HSV infection, the responses of the PBMC and the reconstituted samples were positive while the response to the T-enriched samples was diminished, often to negative values (for example: PBMC 22,936±7640 c.p.m, SI=4·3; T-enriched 3215±946 c.p.m., SI=2·0). Thus there was no evidence that the PBMC, or the T-cell population reconstituted with the adherent cell population, of patients with cutaneous warts, were unable to respond to a viral antigen, other than HPV.

Discussion

The 26 patients assayed had cutaneous warts which were static, improving or had resolved during the study and, in several cases, more than one specimen of blood was obtained from each patient during the course of treatment. Despite the fact that all patients, with one exception, showed at least improvement of their warts, with a smaller number showing resolution, the *in vitro* lymphoproliferative responses to HPV-1 or HPV-2 as antigens were predominantly negative. In contrast, the patients responded well to the mitogen Con A, and those with a clinical history of HSV infections also responded to HSV in an equivalent test. Thus, their T cells' responses were not suppressed generally. Phenotyping of their PBMC showed normal percentage ranges for CD4, CD8, MHC class II, CD1a, CD11b and C57 positive cells.

In the first place, it is pertinent to ask whether a positive response to HPV might be expected in such a group of patients. This depends, principally, on viral

Table 4. Lymphoproliferative responses to HPV-2 in separated PBMC populations of three patients

	44A		34B		46B	
Patient	c.p.m.+SE	SI	c.p.m.+SE	SI	c.p.m +SE	SI
PBMC alone	2940+351		1009+97		2883+504	
PBMC+HPV-2	2587+459	0·9	5037+395	5·0	2933+392	1·0
T-enriched cells alone	310+68		305+14		1194+109	
T-enriched cells+HPV-2	173+65	0·6	384+81	1·2	8902+2866	7·4
Reconstituted cells alone	2919+973		2248+863		3510+348	
Reconstituted cells+HPV-2	4846+1237	1·7	6913+396	3·1	2927+304	0·5
Clinical status	HPV2 Improving		HPV2 Resolved		HPV1 Improving	

c.p.m.+SE, mean count per minute+standard error.
SI, stimulation index.

Table 5. Summary of lymphoproliferative responses to HPV in separated PBMC populations

Clinical group	Antigen	Number of patients in group	Number of patients showing no response by PBMC, or T-enriched cells	Number of patients showing response by PBMC, no response by T-enriched cells	Number of patients showing no response by PBMC, response by T-enriched cells
HPV-1 warts	HPV-1	8	4	1	3
	HPV-2	7	2	1	4
	HSV	3	2	1	0
HPV-2 warts	HPV-1	14	11	1	2
	HPV-2	17	9	1	7
	HSV	14	3	11	0
Normal controls	HPV-1	6	3	0	3
	HPV-2	7	2	0	5
	HSV	4	2	2	0

antigens being expressed in the wart, and on the initiation of an immune response, probably by Langerhans cells interacting with T cells. It is generally believed that HPV infects basal cells of the epidermis following abrasion of the skin, and that expression of early viral genes promotes proliferation and delays maturation of superficial keratinocytes, leading to the characteristic acanthosis and parakeratosis of warts. The control of late gene expression is tightly linked to the state of differentiation of the squamous cells, so that the structural proteins of the virus are only found in the upper layers of the epidermis, the stratum granulosum and stratum corneum. In both HPV-1 and HPV-2 lesions, particularly in the former, many virions are seen, sometimes in crystalline arrays within the nuclei of degenerating keratinocytes in the superficial layers of the stratum corneum.[15] Although the cell bodies of most Langerhans cells tend to be located near the basement membrane, Langerhans cells have long dendritic processes which form a network throughout the epidermis, and play a crucial role in immunosurveillance.[16] Thus, despite most virus particles being produced in the upper epidermis, it is probable that the Langerhans cells are still capable of monitoring their presence and initiating appropriate immune responses. However, two studies have indicated that Langerhans cell numbers are reduced in skin warts,[17,18] and there is a similar picture in cervical HPV lesions and dysplasia, where Langerhans cell numbers are reduced,[19,20] and T-cell numbers are also reduced, with altered subset ratios.[21] The reduction in Langerhans cell numbers may result in ineffective local antigen presentation. HPV may infect Langerhans cells and be cytolytic for them, although this seems unlikely, as HPV particles have never been seen inside Langerhans cells. It is more likely that migration or recruitment of Langerhans cells may be affected, and the interaction between Langerhans cells, HPV-infected keratinocytes and effector T cells within the lesion is unknown. It would be of particular relevance to assay cytokine levels, which may affect migration and function of particular populations of cells, and also adhesion molecule expression.

One of the major difficulties in the study of immune responses in HPV infections has been the lack of an *in vitro* culture system for HPV which would enable preparation of appropriate antigens and target cells. We used viral particles purified from pooled wart parings which were checked by their density on CsCl gradient centrifugation, by electron microscopy and, in the case of HPV-1, by Western blotting. It is probable that some contamination by host cell proteins remains, to which lymphocytes from some subjects may respond. In addition, only the late structural proteins of the virus are present in such a preparation, and the immune response may be generated preferentially to the early proteins which are synthesized at various stages during viral replication. The protein derived from the E4 open reading frame may be particularly important in this context, as it accumulates in the cytoplasm of terminally differentiated keratinocytes where it interacts with the cytokeratin matrix, and can comprise 20–30% of the total cell protein.[22] However, using a fusion protein composed of E4 of HPV-1 or HPV-2 and β-glactosidase expressed in *E. coli*, led to disappointing results, as lymphocytes from most subjects responded to the β-galactosidase itself, to a level indistinguishable from that of the fusion protein. In addition, some of the three-dimensional epitopes of the native protein may be lost on expression as a fusion protein, and these may be crucial for stimulating T-cell responses.

An alternative to fusion proteins is short synthetic peptides, which have been used to test T-cell responses to HPV in subjects without known HPV infections.[23] It was found that no single peptide, representing determinants in HPV-16 L1 and E6, was recognized by all donors and no individual responded to all the peptides. Because in this study stimulation with IL-2 in the presence of the peptides and feeder cells took place before measuring lymphoproliferative responses to individual peptides, it is possible that primary responses may have been generated *in vitro*. This approach has not been used, thus far, in patients with cutaneous warts.

Although only four patients out of 22 in the group where separated PBMC populations were assayed showed a lymphoproliferative response of PBMC to HPV, 16 became positive if the PBMC were first depleted by plastic adherence, and then T cell-enriched by nylon wool adsorption. Five out of seven of the normal control subjects behaved similarly.

Equivalent studies using HSV as antigen did not follow this unexpected pattern. All cell populations were negative in subjects without a clinical history of HSV infection. In subjects with a clinical history of HSV infection, the PBMC showed positive lymphoproliferation to HSV, as did the reconstituted cell population of T-enriched cells plus plastic adherent cells, whereas the T-enriched population alone was either negative or much reduced in its lymphoproliferative response. Thus, it is possible that the adherent cell population contains cells which suppress the lymphoproliferative response to HPV, or that the nylon wool column removes some cell population which inhibits T-cell stimulation by HPV. As can be seen from Figure 3, MHC class II and CD11b-positive cells are not entirely depleted from the T-enriched population by plastic adherence and nylon wool. An alternative method using L-leucine methyl ester had no apparent effect in depleting antigen presenting cells (data not shown).[24]

It is possible that T cells may be subject to an immunoregulatory network in HPV infection, as has been described recently in the T-cell response to human tumours.[25,26] It was shown, for paraganglioma and melanoma, that fresh peripheral blood lymphocytes were not cytotoxic against autologous tumour cells. However, if the lymphocytes were first activated *in vitro* by co-culture with tumour cells in the presence of IL-2 and then cloned, the T cells could be functionally characterized for cytotoxic and regulatory functions. Some T-cell clones amplified cytotoxic T-cell responses, whilst others down-regulated them. Thus, it may be necessary to activate and clone T cells from patients with warts in order to demonstrate the functional properties of the T cells in the response to HPV infection.

Acknowledgment

This work was funded by a grant from the Scottish Home and Health Department.

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British Journal of Dermatology (1992) 127, 560–565.

Effects of cyclosporin A on resident and passenger immune cells of normal human skin and UV-induced erythema reactions

P.PETZELBAUER AND K.WOLFF
Department of Dermatology I, Vienna Medical School, Vienna, Austria

Accepted for publication 21 July 1992

Summary

Cyclosporin A (CyA) has proved effective in various dermatological diseases, but its mechanisms of action within the skin are still ill-defined. In order to characterize more clearly the cellular targets of CyA we examined its effects on skin immune cells in normal and UVB- and PUVA-irradiated skin by means of a three-step immunoperoxidase reaction and immunofluorescence double-labelling technique. The CyA-induced depletion of epidermal Langerhans cells equals that seen with UVB or PUVA alone. CyA alone has no effect on the number and distribution of dermal cells. CyA modulates the UV irradiation-induced changes by: (i) inhibiting the UVB- and PUVA-induced ICAM-1 expression by keratinocytes and (ii) suppressing the PUVA-induced upregulation of CD11a expression by macrophages ($72 \pm 12\%$ of Ki-M8$^+$ macrophages express CD11a with PUVA, compared with $20 \pm 5\%$ with CyA+PUVA, $P<0{\cdot}001$). Neither treatment affected ICAM-1 expression by endothelial cells. In addition, CyA increases PUVA minimal phototoxicity dose from $10 \pm 2{\cdot}6$ J/cm^2 (PUVA alone) to $15{\cdot}3 \pm 3{\cdot}1$ J/cm^2 (CyA+PUVA), ($P<0{\cdot}001$). In conclusion, the effects of CyA on the skin include a down-modulation of the PUVA-erythema reaction, associated with a direct or indirect modulation of adhesion molecule expression.

Cyclosporin A (CyA),[1,3] PUVA,[4] and to a lesser extent UVB[5,6] are effective in clearing psoriatic lesions, and this also applies for a combination of CyA and PUVA.[7] Despite the similarity in clinical response, the immunohistologically detectable effects at the cellular level differ considerably in resolving psoriatic plaques during treatment with CyA and PUVA. The major morphological/phenotypic change in CyA-treated psoriatic plaques is a dramatic reduction in ICAM-1 expression by papillary endothelium,[8] and this contrasts with findings in PUVA-treated psoriatic lesions where endothelial ICAM-1 is not suppressed.[8] However, a CyA-induced resolution of psoriatic lesions is not associated with any change in the density and phenotype of epidermal and dermal leucocytes, at least in the initial phase of treatment, and this is in significant contrast with the marked diminution of the cellular infiltrate induced by PUVA.[8] This indicates that the two treatments act on different or overlapping targets. In order to define more clearly immunohistologically detectable 'target' cells of CyA *in situ* we have examined the effects of CyA on resident and passenger skin cells in normal human skin by means of a three-step immunoperoxidase reaction, and by an immunofluorescence double-labelling technique. In addition, normal skin irradiated with PUVA and UVB was examined by the same procedure, and the effects were compared with those of CyA, CyA+PUVA and CyA+UVB, in order to determine whether CyA modulates UV effects.

Methods

Patients and treatment protocols

Twelve male patients assigned for CyA treatment because of severe plaque psoriasis were included in the study. Their ages ranged from 23 to 50 years, and all were Caucasian (skin type II/III). Informed consent was obtained.

Cyclosporin A. All patients were treated with CyA (Sandimmun, Sandoz AG, Basel, Switzerland), 5 mg/kg/day administered in two equal divided doses. CyA trough levels, monitored twice a week by means of high-performance liquid chromatography, ranged from 30 to 220 ng/ml (mean = 110).

Correspondence: Dr P.Petzelbauer, Department of Dermatology I, Vienna Medical School, Alserstrasse 4, A-1090 Vienna, Austria.

Light sources. The UVB source was a high-pressure mercury lamp (Osram Ultra-Vitalux, Osram GMBH, Munich, Germany). The high-intensity UVA source (Sellas Sunlight 2001, Sellas Med Geräte GMBH, Gevelsberg, Germany) was equipped with a metal halide lamp and a 5 mm window-glass filter. This unit emits a broad peak at 350–400 nm. The UVB contamination was less than 0·01%.

Before initiation of CyA treatment in each of the 12 patients template test fields on non-sun-exposed and clinically normal gluteal skin were exposed to 28, 36, 56, 84 and 112 mJ/cm^2 UVB, and erythema threshold readings (minimal erythema dose, MED) were performed 24 h thereafter. Two hours after ingestion of 5-methoxypsoralen (5-MOP, Geralen®, Gerot Pharmazeutika, Vienna, Austria), 1·2 mg/kg body weight, different template test fields, again on normal gluteal skin, were exposed to 3, 4, 6, 10, 14 and 18 J/cm^2 UVA and threshold erythema (minimal photoxicity dose, MPD) was determined 48 h thereafter. CyA treatment was then commenced, and after 2 weeks of continuous CyA treatment, UVB and PUVA exposures were repeated according to the same protocol, and threshold erythema readings were performed. 5-MOP plasma levels determined at each PUVA test before CyA treatment were comparable with those performed during CyA treatment (mean 49 ± 8 and 58 ± 20 ng/ml, respectively).

Monoclonal antibodies (MAb)

MAbs employed are listed in Table 1. Antibodies were diluted in phosphate-buffered saline (PBS)/bovine serum albumin (BSA) 0·1%, at pH 7·4.

Skin biopsies and immunohistochemistry

Punch biopsies (4 mm) were taken from clinically normal, unirradiated gluteal skin before (controls, $n = 12$) and after 2 weeks of CyA treatment (Group I, $n = 12$) in each patient. In addition, punch biopsies were taken from test sites showing threshold erythema 24 h after UVB irradiation (MED) (Group II, $n = 6$), and 48 h after PUVA irradiation (MPD) (Group III, $n = 6$), respectively. After 2 weeks of CyA treatment, additional biopsies were taken in each patient from gluteal test sites irradiated with a UVB dose (Group IV, $n = 6$) and PUVA dose (Group V, $n = 6$), respectively, identical to that of the first test.

Immunohistochemistry was performed on cryostat sections as previously described,[8] using a three-step immunoperoxidase technique (ABC method; Vector Laboratories, Burlingame, CA, U.S.A.). Double labelling was performed by an immunofluorescence technique at room temperature. Briefly, after pre-incubation with normal sheep serum (diluted 1:10 in PBS/BSA) the first MAbs were visualized by incubation with a rhodamine-labelled second-step Ab ($F(ab')_2$ anti-mouse; An der Grub, Kaumberg, Austria) diluted 1:120 in PBS/BSA. After washing, sections were incubated with normal mouse serum, diluted 1:10 in PBS/BSA. The second MAbs, directly labelled with FITC (anti-Leu 4 and IOT 16), were employed as a 1:10 dilution in PBS/BSA. After incubation for 30 min, followed by washing, slides were mounted with buffered polyvinyl alcohol. Controls were performed by (i) omitting the first MAb, or (ii) by using irrelevant isotype-matched MAb as first-step reagents.

Table 1. Monoclonal antibodies*

Antibody		Dilution	Source‡
CD1a	OKT6	1:1000	OD
CD3	Anti-Leu4	1:800	BD
CD4	Anti-Leu3a	1:100	BD
CD8	Anti-Leu2a	1:100	BD
CD11a	IOT16	1:200	I
CD25	Anti-IL-2R	1:100	BD
CD54	ICAM-1	1:200	I
—	Anti-HLA-DR	1:1000	BD
—	Ki-M8†	1:1000	B

* For main cellular reactivity and membrane components, see Knapp *et al.*[28]

† Broadly reacting anti-macrophage antibody.[29]

‡ OD, Ortho Diagnostic Systems, Inc., Raritan, NJ, U.S.A.

BD, Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.

I, Immunotech S/A, Marseille, France.

B, Behringwerke AG, Marybury, Germany.

Statistical analysis of resident and passenger leucocytes

The number of intraepidermal leucocytes was determined by counting cells within a 3 mm linear length of epidermis, and the density of dermal leucocytes was determined by counting cells in four randomly selected high-power fields (HPF) (0·6 mm in diameter) in each specimen, in at least two different sections (sections were coded so that evaluation was performed in a blinded fashion). The individual values obtained in controls and in each group were then pooled and the mean number of leucocytes was determined. Statistical analysis was performed by using Student's *t*-test. Data are given in mean values ± SD.

Results

Erythema reaction

The PUVA minimal phototoxicity dose (MPD) increased in each patient after 2 weeks of CyA treatment (mean MPD 15·3±3·1 J/cm^2, $n=12$) compared with pretreatment values (mean MPD 10·3±2·67 J/cm^2, $P<0·001$) indicating that CyA suppressed the PUVA erythema reaction. In contrast, CyA had no effect on UVB erythema (Fig. 1).

Epidermal cells

Langerhans cells (LC). Normal epidermis (controls) contained 36·8±9 CD1a$^+$/HLA-DR$^+$ LC per linear mm of epidermis. Numbers of LC decreased significantly in all treatment groups (for absolute numbers and *P* values see Figure 2). The most conspicuous decrease of CD1a$^+$/HLA-DR$^+$ LC was observed with the CyA+PUVA combination, but this was not significant when compared with either CyA ($P=0·06$) or PUVA ($P=0·14$) alone.

Keratinocytes (KC). UVB (Group II, two of six specimens) and PUVA (Group III, five of six specimens) irradiation induced ICAM-1 expression by KC in distinct foci (Fig. 3), whereas in controls and with CyA treatment KC were clearly ICAM-1 negative. When UVB or PUVA were combined with CyA, ICAM-1 expression by KC did not occur (treatment groups IV, V). HLA-DR expression did not occur in any specimen.

T cells. A clustering of a few intraepidermal CD3$^+$/CD11a$^+$ T cells was only found around areas of ICAM-1$^+$ KC, i.e. in two of six UVB and five of six PUVA-irradiated specimens (Fig. 3) but not in any of the CyA treatment groups (I, IV, V). In all groups the ICAM-1$^-$ portions of the epidermis contained extremely low numbers of CD3$^+$ T cells, which did not exceed those found in the controls (0·1 cell/linear mm of epidermis). All CD3$^+$ cells were also CD11a$^+$. The CD4:CD8 ratio was approximately 1:2 in controls and in all treatment groups.

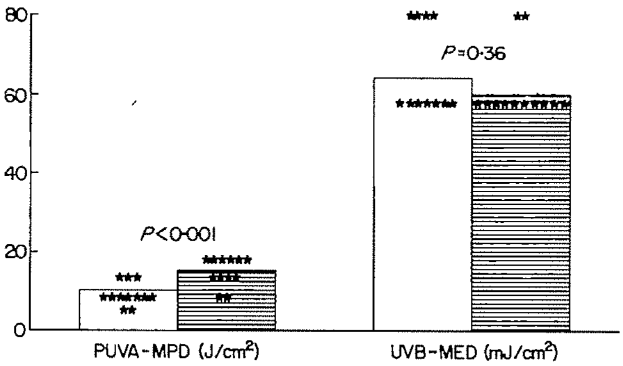

Figure 1. PUVA and UVB erythema threshold before, and after 2 weeks of continuous CyA treatment (bars represent the mean values, asterisks denote the individual values). □, Before CyA; ■, after 2 weeks of CyA.

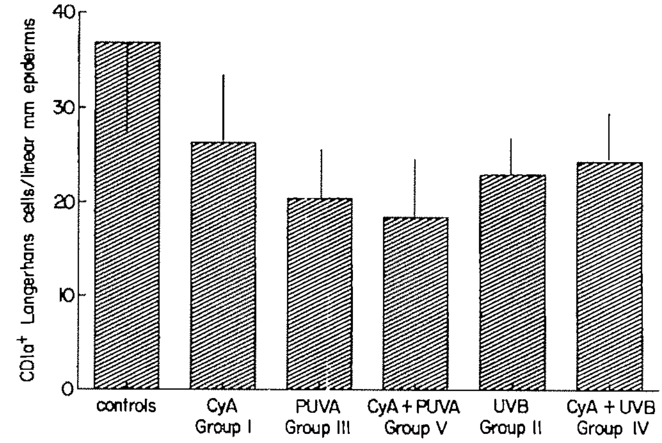

Figure 2. Mean numbers of intraepidermal CD1a$^+$ Langerhans cells/linear mm of epidermis. The decrease in numbers of LC in all treatment groups is significant compared with controls ($P<0·005$ for CyA, UVB and CyA+UVB; $P<0·001$ for PUVA and CyA+PUVA).

Dermal cells

T cells. There were twice the number of perivascular CD3$^+$ T cells in Groups II (UVB) and IV (CyA+UVB) than in controls ($P<0·005$; Fig. 4). Whereas the UVB-induced increase was based on a recruitment of CD4$^+$ cells (CD4/CD8 ratio 2:1), in CyA+UVB-treated skin (Group IV) both CD4$^+$ and CD8$^+$ cells were found in increased numbers (ratio 1:1). In all other treatment groups the numbers of T cells remained unchanged compared with controls (for absolute numbers see Figure 4), the CD4/CD8 ratio being 1:1. Approximately 90% of CD3$^+$ cells were also CD11a$^+$, irrespective of the treatment. Although the mean numbers of dermal CD25$^+$ T cells were reduced in all groups compared with controls, this difference was not significant.

Macrophages. Whereas numbers of dermal Ki-M8$^+$ macrophages and CD1a$^+$ LC remained unchanged in all treatment groups compared with controls (Fig. 4), a significant increase was observed in CD11a$^+$ cells in Groups II (UVB; 43±20 cells/HPF), III (PUVA; 32±

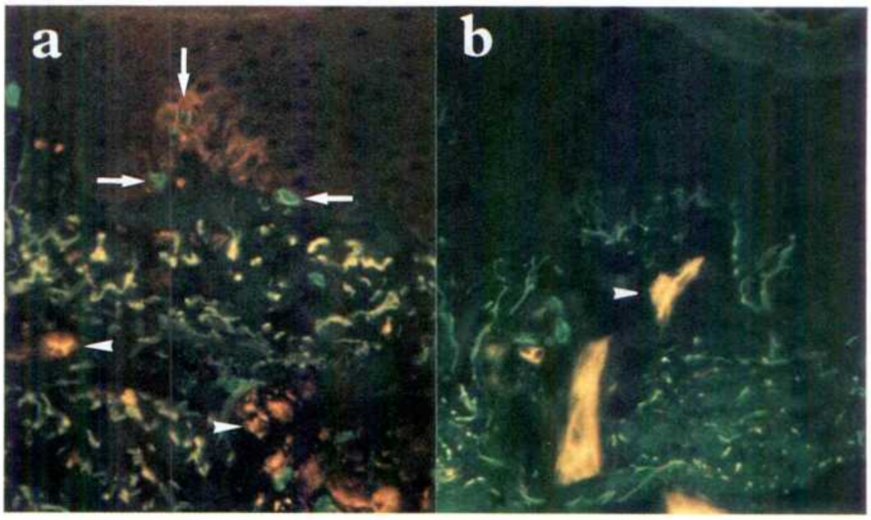

Figure 3. CD3 (green)/ICAM-1 (red) immunofluorescence double staining : (a) PUVA, (b) CyA + PUVA. Note the epidermotropism of CD3$^+$ T cells (green, arrows) within areas of ICAM-1$^+$ keratinocytes (red) in (a). In (b) there are no areas of ICAM-1$^+$ keratinocytes, and clustering of CD3$^+$ T cells is not detectable. Arrowheads point to ICAM-1$^+$ endothelial cells (× 220).

8·7/HPF) and IV (CyA + UVB; 37·7 ± 15/HPF), but not in Groups I (CyA; 17·9 ± 10) and V (CyA + PUVA; 18 ± 5·6) compared with controls (21·8 ± 8; for *P* values see Fig. 4). As evaluated by immunofluorescence double labelling, the increase in the number of CD11a$^+$ cells in Group III (PUVA) was based on a significant increase ($P < 0{\cdot}001$) in the percentage of Ki-M8$^+$ macrophages expressing CD11a (72 ± 12%) compared with 25 ± 7% in controls, 22 ± 4% in Group I (CyA), 20 ± 5% in Group V (CyA + PUVA), 21 ± 6% in Group II (UVB) and 16·3 ± 2·7% in Group IV (CyA + UVB). In Groups II (UVB) and IV (CyA + UVB) the increase in the number of CD11a$^+$ cells compared with controls was the result of an increase of CD11a$^+$/CD3$^+$ T cells (see above).

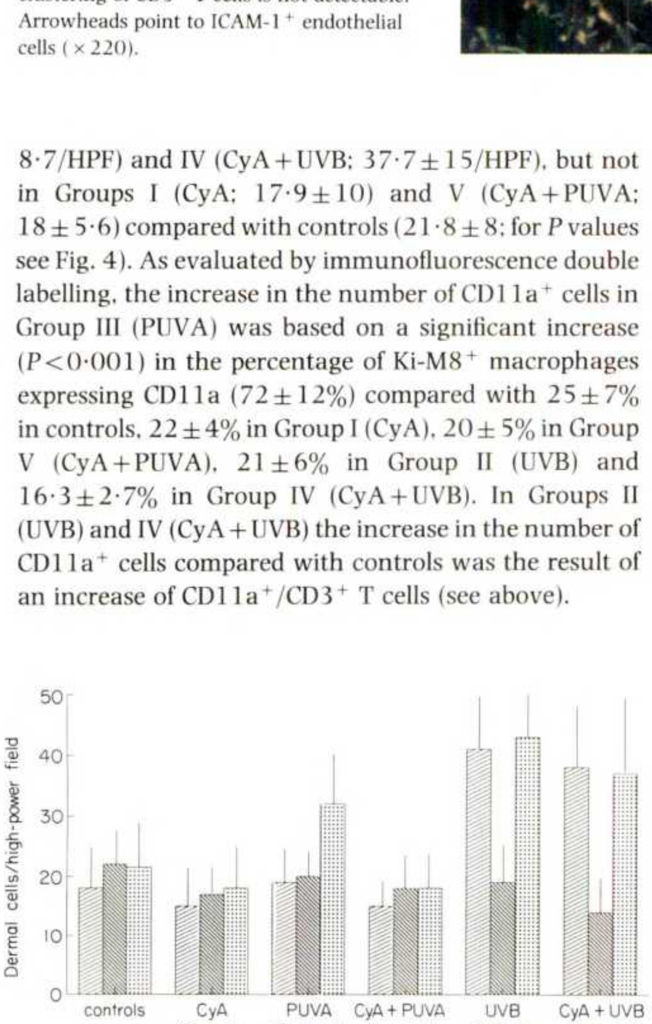

Figure 4. Mean numbers of dermal leucocytes/high-power field. The PUVA-induced increase in dermal CD11a$^+$ cells ($P < 0{\cdot}05$ compared with controls) is not detectable in the CyA + PUVA treatment group ($P < 0{\cdot}01$ compared with PUVA). The UVB-induced increase in CD3$^+$ cells ($P < 0{\cdot}005$ compared with controls) and CD11a$^+$ cells ($P < 0{\cdot}005$ compared with controls) is comparable with that seen in the CyA + UVB treatment group. ▨, CD3; ▧, Ki-M8; ▤, CD11a.

Blood vessels. None of the treatment regimens had any influence on HLA-DR or ICAM-1 expression by dermal endothelial cells compared with controls.

Discussion

The present study demonstrates that PUVA erythema, but not UVB erythema, is inhibited by CyA. At the cellular level it shows that the CyA-induced depletion of epidermal CD1a$^+$/HLA-DR$^+$ LC equals that seen with UVB and PUVA treatment[9,10] and that the combination of CyA with UVB or PUVA does not augment this effect. CyA alone has essentially no effect on the number and distribution of dermal cells in normal skin, but modulates UV irradiation-induced changes in that (i) it suppresses UVB and PUVA-induced ICAM-1 expression by KC and (ii) inhibits PUVA-induced CD11a expression by macrophages.

CyA has proved effective as an inhibitor of a variety of T-cell responses *in vitro*, including antigen-specific proliferation, macrophage-mediated antigen presentation, and antigen- and lectin-induced cytokine production.[11–14] The sparse data available on the *in vivo* effects of CyA on skin cells concern CyA-treated psoriatic lesions,[8,15–17] lichen planus,[18,19] and delayed-type hypersensitivity (DTH) reactions.[20] We,[8] and others,[15,17] have previously shown that CyA leads to a relative increase of epidermal CD1a$^+$ LC in psoriatic lesions where the LC population is usually reduced. We have shown that in normal skin CyA has the opposite effect, in that it depletes the LC population. However, in lichen planus LC have been reported to be unaffected by CyA.[18,19] These divergent findings are difficult to interpret, unless it is assumed that the effects of CyA on LC depend on their state of activation in normal skin and different inflammatory conditions. A functional perturbation of LC can be deduced from the observation that CyA inhibits DTH reactions and T-cell recruitment[20] and, indeed, *in vitro* experiments have clearly demonstrated that CyA decreases the antigen-presenting capacity of LC.[21–23]

Focusing on the effects of CyA on adhesion molecule expression we have recently demonstrated that CyA decreases ICAM-1 expression by endothelial cells in psoriasis lesions during the early phase of lesion regression.[8] Others have found that CyA decreases ICAM-1 expression by KC in psoriasis[15] and in lichen planus.[18] Here we demonstrate that the PUVA-induced increase in ICAM-1 expression by KC, and CD11a expression by macrophages in normal human skin is suppressed by CyA. Although it is possible that CyA accomplishes these effects directly, it is more likely that CyA acts on cytokines regulating the expression of adhesion molecules. Both ICAM-1 and CD11a expression can be induced by certain cytokines, e.g. by interleukin 1 (IL-1), γ-interferon (IFN-γ) and tumour necrosis factor-α (TNF-α).[24–26] Indeed, the *in vitro* effects of CyA include an inhibition of IL-1, TNF-α and IFN-γ[14,27] production. Preliminary experiments in our laboratory, employing *in situ* hybridization of cytokine mRNA on PUVA-irradiated skin sections, have shown a low-level upregulation of TNF-α message in two (but not in six other) specimens, which was not detectable in the CyA + PUVA treated specimens (unpublished observation), suggesting an inhibitory effect of CyA on TNF mRNA production. Finally, it should be noted that UVB irradiation, in contrast with PUVA, does not increase CD11a expression by macrophages, and ICAM-1 expression by KC is only marginally induced. Furthermore, CyA does not inhibit the UVB-induced T-cell recruitment or the UVB-induced erythema reaction, indicating that CyA specifically inhibits a PUVA-induced pathway of activation within the skin. One might speculate that the down-modulation of the PUVA erythema reaction and the inhibition of the PUVA-induced ICAM-1 and CD11a expression by CyA are based on a related mechanism. Animal studies combining PUVA irradiation with antibodies neutralizing cytokine activity (in order to stimulate CyA action) will possibly further characterize this particular CyA effect within the skin.

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British Journal of Dermatology (1992) 127, 566–570.

Mutant p53 oncogene expression in keratoacanthoma and squamous cell carcinoma

T.J.STEPHENSON, J.ROYDS, P.B.SILCOCKS* AND S.S.BLEEHEN†

*Departments of Pathology, *Public Health Medicine and †Dermatology, University of Sheffield Medical School, Sheffield, U.K.*

Accepted for publication 26 July 1992

Summary The tumour suppressor gene p53, located on the short arm of chromosome 17, encodes for a nuclear protein which regulates cell proliferation by inhibiting cells entering S-phase. p53 mutations are alleged to be the commonest genetic abnormality in human cancer. We studied mutant p53 oncoprotein expression, using PAb1801 monoclonal antibody immunohistochemistry, in 25 'ideal' keratoacanthomas and 26 well-, 19 moderately and 18 poorly differentiated squamous cell carcinomas of the skin. While there was a highly significant trend in the proportion of p53 oncoprotein-positive lesions from keratoacanthomas to poorly differentiated squamous cell carcinomas ($\chi^2=17{\cdot}13$, df=1, exact $P=0{\cdot}00003$), p53 expression was inadequate for distinguishing keratoacanthoma from well-differentiated squamous cell carcinoma ($\chi^2=2{\cdot}55$, df=1, exact $P=0{\cdot}18$; corresponding to a sensitivity of 0·84 and a specificity of only 0·36).

It has been proposed that p53 gene mutations are the commonest genetic abnormality in human malignancy.[1] The gene, located on the short arm (p) of chromosome 17, encodes for a nuclear phosphoprotein involved in the inhibition of cell proliferation[2] by preventing cells from entering the S-phase.[3] Combined loss of one allele with mutation of the remaining allele, has been demonstrated at the p53 locus (17p 13·1), a finding typical for suppressor oncogenes.[4] Mutations and deletions of the p53 gene have been demonstrated in several forms of human malignancy,[5–11] including squamous cell carcinomas from internal[12,13] and epidermal sites.[14] The latter study showed p53 mutations at dipyrimidine sites, and the presence of CC to TT double base alterations in epidermal, but not other, malignancies. These are findings typical of UV light-induced DNA damage, and have implications for the aetiology of squamous cell carcinoma of the skin. Present in exons of the p53 gene, the mutations lead to synthesis of an abnormal p53 protein.[4,15] Normal p53 protein regulates cell proliferation and inhibits transformation, but can be converted by point mutations into a possibly dominant oncogene with transforming activity.[16–18]

Owing to the short half-life of the wild-type protein, p53 protein levels in non-transformed cells under normal conditions are sufficiently low to prevent detection by immunohistochemistry.[13] The elevated levels of p53 protein, demonstrable by immunohistochemistry[5,13,19] are thought to result from post-translational stabilization of the mutant protein by complexing with other proteins such as the wild-type product of the normal allele, viral proteins and heat-shock proteins.[16,18,20]

Immunohistochemistry for p53 protein is a valid screening method for mutations in the p53 gene, because previous combined immunohistochemical and molecular biological studies in various human tumours have shown strong correlation between p53 gene mutations and immunohistochemical staining for p53 protein.[13,19,21,22]

We selected monoclonal antibody PAb1801 (Cambridge Research Biochemicals, U.K.), which recognizes an epitope between amino acids 32–37 close to the N-terminus of both wild-type and mutant p53 protein,[23] with which to perform immunohistochemistry. This highly specific monoclonal antibody reliably recognizes p53 protein; other antibodies (PAb240, PAb241) offer no advantage in recognizing mutant rather than wild-type p53 protein in human tissues.[21,24,25] Furthermore, the effect of different fixation processes on immunohistochemical demonstration and localization of p53 protein by this antibody is known.[19]

We compared the expression of mutant p53 protein in keratoacanthomas, and squamous cell carcinomas with various degrees of differentiation, for three reasons:

Correspondence: Dr T.J.Stephenson, Department of Pathology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, U.K.

(i) because of continuing interest in pathological differences between keratoacanthoma and squamous cell carcinoma;[26,27] (ii) because keratoacanthomas are an example of an invariably regressing neoplasm,[28] and analysis of p53 mutations in these lesions may add to the still-evolving story[19] of how far down the multistep pathway to neoplasia p53 mutation lies; (iii) if keratoacanthomas express mutant p53 protein, then either the p53 mutation is reversible or is not a determinant of obligate malignancy. This would be an interesting finding in the light of the recent demonstration[28] of *H-ras* oncogene in rabbit keratoacanthomas and its implication in the process of tumour regression.

Methods

Selection of cases

Proof that a keratoacanthoma is correctly diagnosed as such is a perennial difficulty in this type of study.[29] As our 'gold standard' keratoacanthomas, we selected 25 in which the clinical presentation, naked eye and histological features were absolutely typical.[30] For comparison, 26 well-, 19 moderately, and 18 poorly differentiated squamous cell carcinoma excisions were selected by review of the departmental classified files.

Immunohistochemistry

Tissues were fixed for 12–36 h in 10% neutral-buffered formalin, and embedded in paraffin wax. One section was cut from each block for diagnostic review, and the next two sections were taken for the avidin–biotin complex (Vector Laboratories, U.K.) immunoperoxidase technique. The primary antibody, PAb1801, was applied at 1:400 dilution for 1 h at 20°C. Negative immunohistochemical controls were performed by omitting the primary antibody and substituting other monoclonal antibodies of the same immunoglobulin subtype. Normal skin and benign adnexal neoplasms were used as negative tissue controls. Positive tissue controls included prostatic adenocarcinomas which, as fresh tissue, had stained with the antibody PAb240, which is thought to be specific for mutant as opposed to wild-type p53 oncoprotein.[31] The same two observers (T.J.S. and J.R.) viewed each case simultaneously, blind as to the histological diagnosis. Where any tumour staining was seen—either nuclear, or nuclear and cytoplasmic were accepted as genuine[19]—the case was assigned to the positive category.

Statistical analysis

χ^2 tests on the proportion of p53 positive lesions were performed using the package STATXACT[32] which yields exact *P* values even with small sample sizes.

Results

In all cases in which staining was detected, this was nuclear, with the exception of two cases of poorly

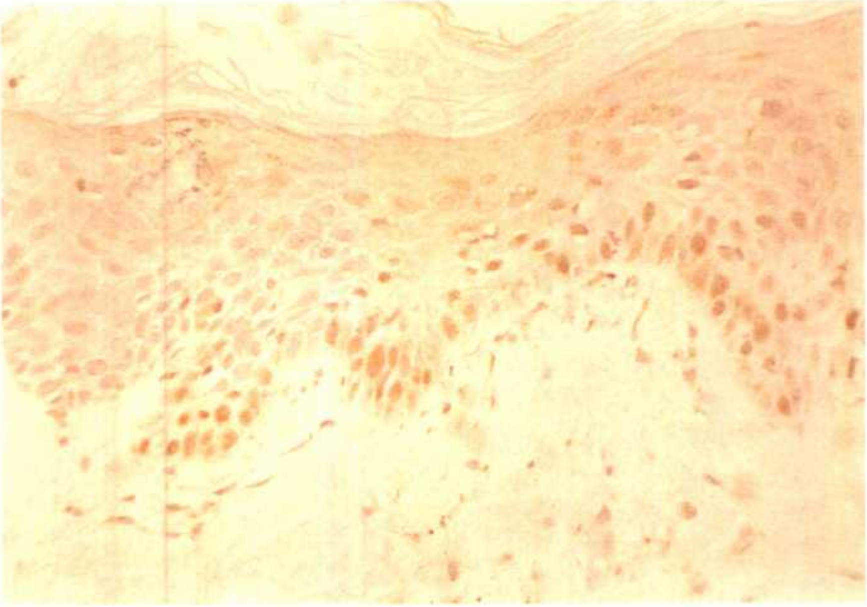

Figure 1. Tumour/stroma interface of a keratoacanthoma at high power. Brown-stained nuclei can be seen in the basal layers of the epithelium. (Immunoperoxidase for p53 protein with haematoxylin counterstain.)

Table 1. Mutant p53 immunohistochemistry: all lesions

		Squamous cell carcinoma: degree of differentiation		
	Keratoacanthoma	Well	Moderate	Poor
p53 negative	21	14	9	4
p53 positive	4	8	10	14
Odds ratio	1	0·33	0·17	0·05

χ^2 (trend), 1 degree of freedom = 17·13, $P < 0·00003$.

Table 2. Mutant p53 immunohistochemistry: the two most easily confused lesions

	Keratoacanthoma	Well-differentiated squamous cell carcinoma
p53 negative	21	14
p53 positive	4	8

Sensitivity of p53 immunostaining negative/positive in detecting keratoacanthoma = 0·84

Specificity of p53 immunostaining negative/positive in detecting keratoacanthoma = 0·36

χ^2 (1 degree of freedom) = 2·55; $P = 0·18$ (not significant).

differentiated squamous cell carcinoma which also showed faint cytoplasmic staining. In the four keratoacanthomas showing staining, this was in nuclei of cells in the deep part, or the peripheral advancing margin of the tumour (Fig. 1). In the 32 squamous cell carcinomas with positive staining, this was in nuclei more widely distributed within the neoplasm (Fig. 2).

Table 1 shows the profile of mutant p53 protein immunohistochemistry for all lesions. Four of 25 keratoacanthomas, 8 of 22 well-, 10 of 19 moderately, and 14 of 18 poorly differentiated squamous cell carcinomas showed mutant p53 protein expression. There was, therefore, a trend for keratoacanthomas to be negative, and for progressively less differentiated squamous cell carcinomas to be positive (exact $P = 0·00003$). However, if p53 protein immunohistochemistry were applied as a 'test' to differentiate between keratoacanthoma and well-differentiated squamous cell carcinoma—the main differential diagnosis[30]—this would not be successful ($\chi^2 = 1·55$, df = 1, exact $P = 0·18$) (Table 2). This corresponds to a sensitivity of p53 protein expression negative/positive in detecting keratoacanthoma of 0·84 with a specificity of only 0·36.

Discussion

Loss or alteration of the 54-kDa nuclear phosphoprotein encoded by the p53 gene,[16] which is involved in suppression of cell proliferation, is thought to contribute to the deregulated proliferation of neoplastic cells.[4] p53 mutations, sometimes accompanied by chromosome 17

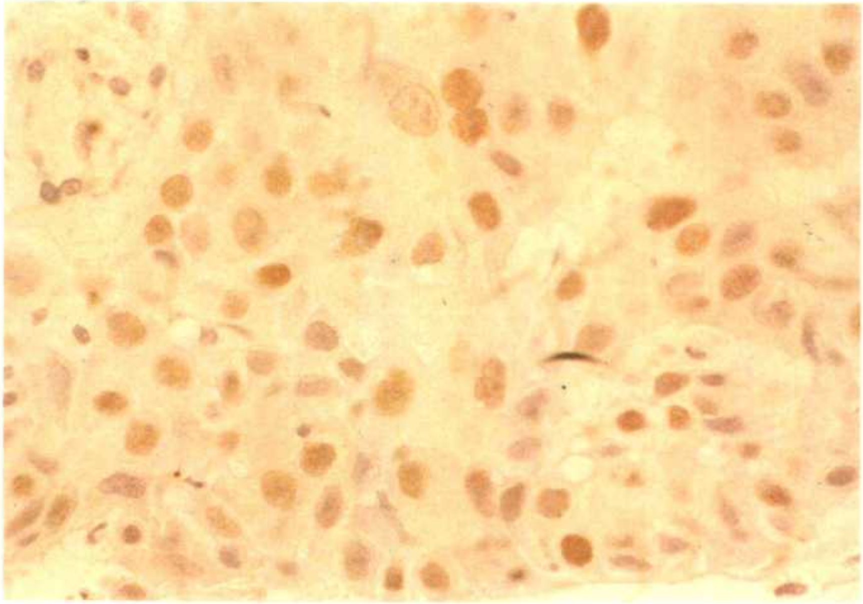

Figure 2. Centre of a poorly differentiated squamous cell carcinoma at high power. The brown-stained nuclei extended throughout the tumour. (Immunoperoxidase for p53 protein with haematoxylin counterstain.)

deletions, were discovered in cancer of the colon,[15] and subsequently described in breast cancer,[6,24] astrocytoma,[4] lung cancer[13] and leukaemias.[9,10] These findings have led to the suggestion that p53 mutations are the commonest genetic abnormality in human cancer.[1]

Although there are cases of immunohistochemical detection of p53 protein in non-neoplastic conditions of lymphoid tissues, indicating non-mutational stabilization of the p53 protein,[11] in neoplasia the immunohistochemical detection of p53 protein has been shown to be synonymous with p53 gene mutations.[13,21,22]

We have demonstrated p53 protein, and by implication p53 mutation, in a limited number (four of 25) of clinically and histopathologically typical[30] keratoacanthomas. Provided that these cases are not misdiagnosed squamous cell carcinomas—a misdiagnosis which is difficult to eliminate[29]—then we have shown, by implication, p53 mutation in a 'neoplasm' which is invariably subject to spontaneous regression.[28] In addition, although the number of positive keratoacanthomas is small, we have shown a difference in distribution of the p53 protein-containing nuclei between the lesions. In keratoacanthomas, the stained nuclei were in the tumour immediately adjacent to the surrounding stroma (Fig. 1) whereas in squamous cell carcinomas nuclear staining was more widely distributed (Fig. 2).

If p53 gene mutations occur in the spontaneously regressing keratoacanthoma, this is a surprising finding, in view of the proposed relatively late occurrence of p53 gene mutations during the multistep pathway to neoplasia.[19] Furthermore, if p53 gene mutation occurs in keratoacanthomas, then this must in some way be either reversible, or not an invariable precursor of malignancy. In our small group of positive keratoacanthomas, the p53 protein-expressing nuclei were at the peripheral (possibly 'growing') edge of the tumour and not at the central area near the crater, where regressive features are common.[30] In squamous carcinomas, which are normally relentlessly progressive and lack regressive features, p53 protein expression was uniform throughout the tumour. Other authors have reported a tendency for well-, but not poorly differentiated, squamous cell carcinomas to have selective p53 protein expression at the periphery.[33]

The reason for spontaneous regression in keratoacanthomas is unknown, although this may involve cell-mediated immunity[34,35] or recapitulation of the normal growth and involution phases of the hair follicle from which some consider it arises.[36,37] Of additional interest to our finding of a selective distribution of p53 protein expression in keratoacanthomas is the demonstration, in a rabbit keratoacanthoma model, of *H-ras* oncogene and its implication in tumour regression.[28]

While p53 mutations can include those typical of UV-induced DNA damage,[14] p53 mutations occur in squamous carcinomas of internal[12,13] as well as epidermal[14] sites. In internal sites, UV-damage is not likely to be a carcinogen. Our demonstration of mutant p53 protein in keratoacanthomas and squamous carcinomas carries no implication for the aetiology because from a protein-based immunohistochemical study we cannot deduce the type of genetic mutations occurring.

With regard to the popular topic of comparing attributes of well-differentiated squamous cell carcinoma and keratoacanthoma,[30,35] we were not surprised that our study showed no clear-cut distinction (Table 2) between the two lesions. Our previous work,[26,27,34] and that of others[34] leads us to believe that keratoacanthoma and well-differentiated squamous cell carcinoma have more pathological similarities than differences. Their radically different behaviour awaits an explanation.

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British Journal of Dermatology (1992) 127, 571–574.

Tenascin expression in basal cell carcinoma

A.A.VERSTRAETEN,*† E.J.MACKIE,‡ PH.C.HAGEMAN,† J.HILGERS,* D.J.SCHOL,* G.J.DE JONGH§ AND J.SCHALKWIJK§

**Department of Obstetrics and Gynaecology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands*

†Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

‡Rheumatology Research Unit, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, U.K.

§Department of Dermatology, University Hospital Nijmegen, Rene Descartesdreef 1, 6500 HB Nijmegen, The Netherlands

Accepted for publication 12 June 1992

Summary

Tenascin (hexabrachion, cytotactin) is an extracellular matrix glycoprotein whose expression is strongly increased in hyperproliferative skin diseases, as shown by immunohistochemistry with polyclonal sera. In this study we describe a new monoclonal antibody (T2H5) against human tenascin. The specificity of T2H5 was validated by sequential immunoprecipitation of tenascin with polyclonal sera. T2H5 was used to analyse the presence of tenascin in basal cell carcinoma. Using Western blotting, at least two forms of tenascin were found, with approximate molecular weights of 210 and 300 kDa. In cultured human skin fibroblasts only the high molecular weight form was found.

Tenascin (TN, which is identical to hexabrachion[1] and cytotactin[2]) is an extracellular matrix molecule with restricted expression in adult human tissues.[3] It is abundantly expressed during embryogenesis, and re-expression can be found during wound healing and in tumour stroma.[4–8] In normal human skin tenascin is sparsely distributed.[9–12] Its expression is restricted to the papillary dermis, and the dermal fibroblasts are the most likely source. Tenascin is not found in the epidermis. We have shown that in a number of hyperproliferative skin diseases (psoriasis, skin tumours) tenascin expression is strongly increased in the dermis.[10] Recently, we found that dermal tenascin expression is linked to epidermal hyperproliferation following tape stripping,[12] suggesting a role of the epidermal compartment in the regulation of tenascin expression. Previous data on tenascin in human skin were largely obtained with polyclonal sera against tenascin from various animal sources, using immunohistological techniques.[7–9] In this study we describe a monoclonal antibody against human tenascin, and its application to human skin on Western blots. It is shown that in basal cell carcinoma two forms of tenascin can be found, in contrast with normal skin and cultured fibroblasts where only one predominant form is found.

Correspondence: Dr J.Schalkwijk.

Methods

Polyclonal antisera

Antiserum against tenascin purified from rat embryo and chicken fibroblast cultures was raised in rabbits, and purified as described previously.[6]

Preparation of immunogen and generation of monoclonal antibodies

A human mammary tumour specimen, obtained at surgery, was homogenized on ice in PBS containing protease inhibitors, using a Polytron PTA 10-35 homogenizer (Kinematica, Lucerne, Switzerland) for 60 s at maximum speed, cleared by centrifugation and stored at −20°C until used. Protein concentration was measured using the BioRad Protein Assay kit. Immunocompetent murine splenocytes were obtained by immunizing (intraperitoneal) BALB/c mice at weekly intervals for 3 weeks with 30 μg tumour homogenate. Three weeks after the last immunization the mice were boosted with ± 50 μg homogenate intravenously on the third, fourth and fifth day before cell fusion. Splenic lymphocytes were fused with Sp-2/0-Ag 14 non-secreting myeloma cells, and cultured according to standard protocols. The hybridoma supernatants were screened by indirect peroxidase immunohistochemistry on cryostat sections of human tissues.

Mouse monoclonal anti-chick tenascin, M1[13] which does not react with mammalian tenascin was used as a negative control.

Immunoprecipitations and Western blotting

Cells of a human osteosarcoma cell line (Saos-2) were grown in medium containing 10% fetal calf serum. Medium was collected every 2 days from confluent cells and concentrated 100-fold by precipitation with 40% saturated ammonium sulphate, followed by dialysis against PBS. For immunoprecipitations, 20-μl aliquots of concentrated Saos-2-conditioned medium (SCM) were incubated with 20 μl normal rabbit serum (NRS; for samples to be precipitated with anti-TN or NRS) or M1 ascites (for samples to be precipitated with T2H5 or M1) for 30 min; subsequently 40 μl protein G Sepharose (Pharmacia), suspended in 20 μl PBS, was added and incubated for 30 min. Samples were centrifuged and 30-μl aliquots of the supernatants incubated with 10 μl anti-TN, normal rabbit serum, T2H5 or M1 ascites, for 30 min, and subsequently with 40 μl protein G Sepharose for 30 min. The samples were centrifuged and the precipitates washed three times in PBS, and boiled in electrophoresis sample buffer. The supernatants were taken through a second cycle of precipitation with antibody, protein G Sepharose, and centrifugation. These supernatants and the precipitates from the first cycle of immunoprecipitation were run on 5% SDS polyacrylamide gels under reducing conditions, and blotted on nitrocellulose using the Biorad Mini-gel and Transblot systems. Proteins were detected on the blots with anti-TN or T2H5, followed by alkaline phosphatase-labelled anti-rabbit immunoglobulin or anti-mouse immunoglobulin, respectively.

Tissue processing

Basal cell carcinomas from three patients were obtained after surgery at the out-patient Department of Dermatology, Academic Hospital Nijmegen, The Netherlands. Tissue specimens were snap frozen in liquid nitrogen and stored at −80°C until further use. For extraction of tenascin, the tissues were minced with a razor blade and homogenized using a glass–glass grinder in a solution containing 0·2 M ethanolamine, 7 M urea, 6 mM dithiothreitol and 2 mM PMSF, pH 11·3. The homogenate was extracted at 4°C for 72 h. After centrifugation, the high-molecular-weight proteins were precipitated from the supernatant by the addition of ammonium sulphate (final saturation 40%). After centrifugation at 12,000 *g* for 10 min, the pellet was redissolved in SDS–PAGE sample buffer. SDS–PAGE (7·5% gel) and Western blotting were performed as described above.

Fibroblast cultures

Normal human skin fibroblasts were cultured according to standard procedures. The supernatants of first passage cultures were used as a source of tenascin. High-molecular-weight proteins were obtained by ammonium sulphate precipitation, and analysed on Western blots as described above.

Results

Characterization of monoclonal antibody T2H5

Culture supernatants of hybridomas were screened by indirect immunoperoxidase histochemistry on frozen sections of human breast tumours and normal mammary glands for their reactivity with tumour stroma (not shown). Two monoclonal antibodies, T2H5 and T3C3, were selected which showed histological reaction patterns similar to polyclonal anti-TN antiserum (MA-3); these clones were further stabilized. The monoclonal antibody T2H5 was characterized in detail, and used for further studies. In order to establish the specificity of the antibody, sequential immunoprecipitation was performed, and analysed on Western blots as described in the Methods. On immunoblots of conditioned medium from human osteosarcoma cells (SCM) T2H5 and polyclonal anti-TN both reacted with a single band migrating in the same position (Fig. 1, lanes 1 and 2). The size of this band was estimated to be about 330 kDa, when appropriate high-molecular-weight standards were used, as described by Lightner *et al.*[9] After SCM was incubated with NRS, T2H5 still detected a band in this position (Fig. 1, lane 3), but after precipitation with anti-TN the band was no longer detectable (Fig. 1, lane 4). Similarly, reactivity with anti-TN was significantly depleted by incubation with T2H5 (Fig. 1, lane 6), compared with SCM which had been incubated with the control M1 antibody (Fig. 1, lane 5). The tenascin band was not completely removed by two cycles of precipitation with T2H5, perhaps because of the known lower affinity of protein G for mouse immunoglobulin than for rabbit immunoglobulin. T2H5 reacted with a band precipitated by anti-TN (Fig. 1, lane 7) but not by NRS (Fig. 1, lane 8) and anti-TN reacted with the protein precipitated by T2H5 (Fig. 1, lane 9) but not by the control M1 antibody (Fig. 1, lane 10).

Detection of tenascin in basal cell carcinoma

Previous studies showed that basal cell carcinomas are positive for tenascin using immunohistological techniques with polyclonal sera. Figure 2 shows that in 7 M urea extracts of these tissues tenascin can be demonstrated on Western blots, both with a polyclonal serum (lane 2) and with the well-characterized monoclonal antibody T2H5 (lane 3). Both sera recognize two bands. The low-molecular-weight band is approximately 210 kDa. The value for the high-molecular-weight band was estimated to be about 300 kDa. T2H5 also recognizes a high-molecular-weight band at approximately the same position in culture supernatants of human skin fibroblasts (lane 1). Control sera were negative (not shown).

Discussion

In this article we describe a new monoclonal antibody against human tenascin, and show its application to skin. We have previously demonstrated with immunohistology that tenascin expression is grossly increased in the connective tissue of hyperproliferative skin diseases, including basal cell carcinoma.[10] In the present study we give qualitative data on tenascin in basal cell carcinoma. Using Western blots two distincts bands can be detected in 7 M urea extracts of skin specimens obtained after surgery. Whether these different forms of tenascin are the result of alternative splicing as shown in various other tissues,[2,14] or proteolytic breakdown, is not clear. Because tenascin is very difficult to extract from the tissues, we have no information on whether the relative amounts of extracted protein actually reflect the amounts found *in vivo*.

The molecular weights given here, and in the literature, are only approximate figures. Most studies on tenascin from various sources report values between 180 and 260 kDa. Lightner *et al.*[9] recently reported that in normal human skin one predominant form of approximately 330 kDa could be found, both with monoclonal and polyclonal antisera. In basal cell carcinoma and in cultured fibroblasts we have found a band of approxi-

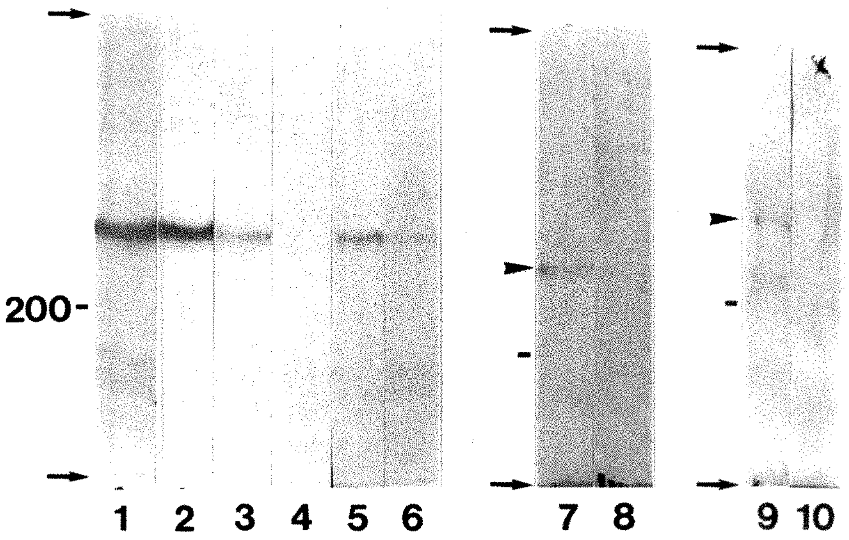

Figure 1. Immunoprecipitations of concentrated Saos-2 (human osteosarcoma cells) conditioned medium (SCM) with monoclonal antibody T2H5 and polyclonal rabit anti-rat tenascin antiserum (anti-TN). All lanes show immunoblots of 5% SDS polyacrylamide gels, run under reducing conditions. Blots from three separate gels are shown. For each gel a bar indicates the position of myosin (about 200 kDa), arrows indicate origin and front, and for the last two gels an arrowhead indicates the position of migration of the band in SCM that reacted with anti-TN. Lanes 1 and 2, SCM reacted with anti-TN (lane 1) or T2H5 (lane 2). Lane 3, supernatant after immunoprecipitation of SCM with normal rabbit serum (NRS), blotted and reacted with T2H5. Lane 4, supernatant after immunoprecipitation of SCM with anti-TN, blotted and reacted with T2H5. Lane 5, supernatant after immunoprecipitation of SCM with M1 (a monoclonal anti-chick tenascin that does not cross-react with mammalian tenascin), blotted and reacted with anti-TN. Lane 6, supernatant after immunoprecipitation of SCM with T2H5, blotted and reacted with anti-TN. Lane 7, precipitate after immunoprecipitation of SCM with anti-TN, reacted with T2H5. Lane 8, precipitate after immunoprecipitation of SCM with NRS, reacted with T2H5. Lane 9, precipitate after immunoprecipitation of SCM with T2H5, reacted with anti-TN. Lane 10, precipitate after immunoprecipitation of SCM with M1, reacted with anti-TN.

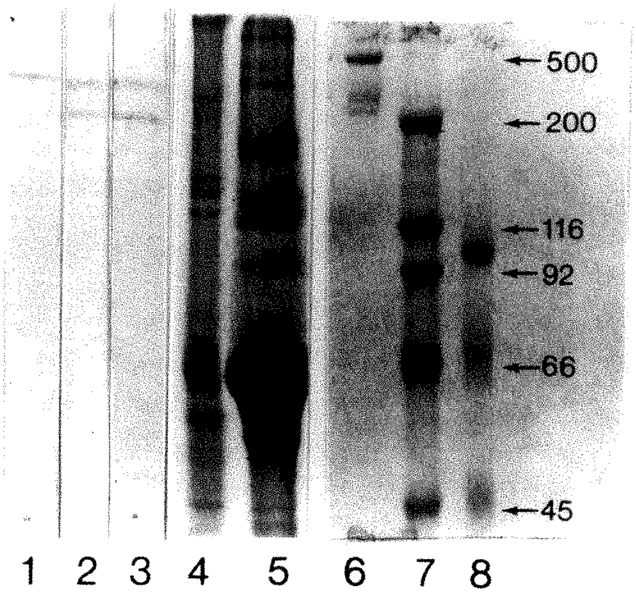

Figure 2. SDS-PAGE and Western blot of fibroblast culture medium and basal cell carcinoma extract. Lane 1, fibroblast culture medium stained with T2H5. Lanes 2 and 3, basal cell carcinoma extract stained with T2H5 and polyclonal anti-TN serum. Lanes 4 and 5, high-molecular-weight preparations of fibroblast culture medium and basal cell carcinoma extract, total protein staining with Coomassie. Lane 6, non-reduced plasma fibronectin. Lane 7, high-molecular-weight markers. Lane 8, prestained molecular weight markers.

mately 300 kDa. However, in basal cell carcinoma an additional band of equal intensity (approximately 210 kDa) is found. The biological significance of this finding is not clear at present.

The monoclonal antibody described in this study was also used in immunohistochemical studies. In various skin biopsies (including basal cell carcinoma) a staining pattern was found which was similar to that described previously[10] using polyclonal antisera on frozen sections (data not shown).

We are currently comparing the expression pattern of tenascin in wound healing and psoriasis with that of normal skin using Western blotting and *in situ* hybridization. This could lend support to the hypothesis that expression of the additional 210-kDa form is related to epidermal activation or disturbance of cutaneous homeostasis. Using cDNA probes for the alternatively spliced tenascin segment, we can also obtain information on whether the different forms of tenascin (as seen on Western blots) are derived from different transcripts, or whether they are generated by proteolytic cleavage during the extraction procedure. Clearly these issues need further investigation, and could shed light on the role of the enhanced expression of tenascin in wound healing and certain skin diseases.

Acknowledgments

The authors wish to thank M.van Doornewaard and D.Atsma for excellent technical assistance, and Dr J.H.Daams for valuable advice.

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British Journal of Dermatology (1992) 127, 575–579.

Density and morphology of Langerhans cells in basal cell carcinomas of the face and trunk

L.BERGFELT, O.LARKÖ AND M.LINDBERG*
Department of Dermatology, University of Gothenburg, Gothenburg, Sweden
**Department of Dermatology, University Hospital, Uppsala, Sweden*

Accepted for publication 9 June 1992

Summary

We investigated the density and morphology of Langerhans cells in epidermal sheets of basal cell carcinomas in chronically sun-exposed skin (face) and less exposed skin (trunk) of 65 patients. Langerhans cells in perilesional and control skin at the same anatomical sites as the tumours were also examined. Two markers (ATPase and OKT6) were used in a parallel fashion to identify Langerhans cells. The density of the cells was reduced, and their morphology was changed in epidermis overlying tumours of both the face and trunk. These alterations were confined to tumour areas, as Langerhans cells in perilesional skin were normal when compared with control skin at both anatomical sites. Results with both markers were the same.

The epidermal Langerhans cell (LC) functions as an antigen-presenting cell in contact hypersensitivity reactions.[1,2] It has also been suggested that the LC is involved in the initiation of immune responses to neoantigens in skin tumours.[3,4] UV radiation reduces the number of detectable LCs in the epidermis and changes their morphology.[5] Depletion of the immunocompetent LCs has been linked to the development of UV-induced skin cancer,[4,6] and might precede the development of basal cell carcinoma (BCC). This skin tumour arises mainly on sun-exposed areas, but can also occur on covered skin.[8,9] In a number of studies, using different staining techniques, the density of LCs in basal cell carcinoma has been compared with their density in normal skin. However, the results have been conflicting.[7,10–14]

To evaluate the relation between UV radiation, LCs and basal cell carcinoma, we examined biopsies from a large number of BCCs on chronically sun-exposed skin (face) and on more protected skin (trunk). The number and morphology of LCs in the epidermis overlying BCCs was compared with those in perilesional and/or control skin from the same patient, taken from the same anatomical site as the tumour. Two different staining procedures were employed for LC detection.

Methods

Subjects

Sixty-five patients, (37 female, 28 male) with basal cell carcinomas were included in the study. In six patients more than one BCC was examined. The patients were all Caucasians, with a mean age of 70 years (range 24–90). They were divided into different groups with regard to tumour location (face or trunk) and clinical subtype (superficial or nodular BCC). After intradermal injection of local anaesthetic (Xylocaine®), 3-mm punch biopsies were obtained from tumour-bearing skin, perilesional and/or control skin from the contralateral side of the body, in the same anatomical region as the tumour. The diagnosis of BCC ($n = 71$) was confirmed by histological examination of paraffin-embedded sections stained with hematoxylin and eosin.

The study was approved by the local ethical committee.

Staining techniques

The skin samples were incubated for 2 h in EDTA. After incubation the epidermis was separated from the dermis and divided into halves. One part was fixed in acetone for 20 min. The other half was fixed in formaldehyde-cacadylic acid solution at 4°C overnight, and then stained for ATPase activity.[15] Acetone-fixed specimens

Correspondence: Dr L.Bergfelt, Department of Dermatology, University of Gothenburg, S-413 45 Gothenburg, Sweden.

were washed in PBS and incubated in OKT6 (DAKO-T6, code M 721, Dakopatts, Denmark) in PBS (1:50 dilution) at 4°C overnight. After incubation with the primary antibody, the epidermal sheets were washed in PBS and incubated with peroxidase-conjugated rabbit anti-mouse antibody (P161 Dako-Immunoglobulins, Copenhagen) for 30 min at 37°C. After incubation, the specimens were washed in PBS and developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, MO) 6 mg in 10 ml 0·05 M Tris buffer with addition of 3% hydrogen peroxide for 5 min, and mounted dermal side up in Mountex mounting medium.[16]

Counting of Langerhans cells

ATPase-positive and OKT6-positive cells were examined by light microscopy. The mean number of LCs/mm^2 was determined by counting 5–15 random fields at ×400 magnification using an optical grid. By focus adjustment, LCs at different levels in the epidermis were evaluated.

Statistical analysis

The differences in the number of Langerhans cells in the epidermis between tumour and perilesional skin, tumour and control skin and perilesional and control skin were calculated for each staining technique in each specimen. Analysis of variance was used to evaluate the influence of age, sex, body location and clinical classification on each of the calculated differences. Student's *t*-test for paired observations was used to analyse the differences in the number of positive cells obtained with the two staining techniques in all biopsies, and to analyse the differences between tumour and perilesional, tumour and control, and perilesional and control skin for each staining technique, clinical classification and body location.

Table 1. The number of positive cells/mm^2 with each staining technique in tumour, perilesional and control skin (for all biopsies)

Staining	Skin area	Number of biopsies	Min.	Max.	Mean
ATPase	Control	64	411	968	686
	Perilesional	49	425	1012	708
	Tumour	70	262	882	506
OKT6	Control	42	403	812	640
	Perilesional	32	450	838	626
	Tumour	46	162	677	426

Results

The mean numbers of ATPase-positive and OKT6-positive cells/mm^2 in the epidermal sheets of tumours, perilesional and control specimens are presented in Table 1. There was no statistically significant difference between the two staining methods. Compared with perilesional and control skin, the number of positive cells/mm^2 was significantly reduced ($P<0\cdot05$) in the epidermal component of BCC, for both ATPase and OKT6 (Table 2).

The analysis of variance demonstrated that neither clinical classification, body location, age, nor sex influenced the significant differences in the number of positive cells/mm^2 in tumour-bearing skin compared with perilesional and control skin. Table 3 shows the number of ATPase$^+$ and OKT6$^+$ cells/mm^2 for both clinical classifications, and at both body locations.

The decrease in the number of LCs in the epidermis overlying BCCs was associated with striking morphological changes. The LCs were more rounded than normal, with no dendrites, or short, clumsy dendrites (Fig. 1). Some of the LCs had a single elongated dendrite without branching (Fig. 2). The altered morphology of LCs was not found in perilesional or control skin. In

Table 2. The differences in the number of positive cells/mm^2 in epidermis between control and tumour, control and perilesional, and tumour and perilesional skin for each staining technique

Staining	Difference	Number of patients	Mean	SD	*t*-test
ATPase	Control–tumour	63	−181·4	125·1	$P<0\cdot05$
	Control–perilesional	42	−17·2	94·2	NS
	Tumour–perilesional	48	−185·9	139	$P<0\cdot05$
OKT6	Control–tumour	42	212·5	164	$P<0\cdot05$
	Control–perilesional	28	20	66·9	NS
	Tumour–perilesional	32	−201·4	50	$P<0\cdot05$

SD, standard deviation. NS, not significant.

Table 3. The number of positive cells/mm^2 in tumour, perilesional and control skin for each body location, clinical classification and staining technique

Body location	Clinical classification	Skin area	Stain	Number of biopsies	Mean	SD
Face	Nodular	Control	A	22	693	125
			P	15	663	60
		Perilesional	A	17	699	153
			P	11	631	80
		Tumour	A	27	495	139
			P	19	432	149
	Superficial	Control	A	5	631	173
			P	3	617	184
		Perilesional	A	3	704	61
			P	2	630	78
		Tumour	A	6	516	190
			P	3	429	63
Trunk	Nodular	Control	A	9	697	134
			P	6	684	118
		Perilesional	A	6	760	141
			P	4	585	129
		Tumour	A	9	496	216
			P	6	443	137
	Superficial	Control	A	27	683	117
			P	17	604	114
		Perilesional	A	23	703	153
			P	15	632	90
		Tumour	A	27	519	155
			P	17	406	129

A, ATPase staining; P, OKT6-peroxidase staining, SD, standard deviation.

several tumour specimens, areas with aggregates of rounded, darkly stained cells, identified as LCs with both ATP-ase and OKT6, were intermingled with areas that had a low LC density.

Discussion

The density and morphology of Langerhans cells in the epidermal component of BCCs and normal skin have been compared in several studies.[7,10–14] The results have varied between the studies, and both increases and decreases in the number of LCs in epidermis overlying BCCs have been reported. In previous studies, the number of BCCs has been small, and in most studies vertical skin sections were used.[3,10–14] Control specimens were not always available,[7] or were obtained from another location than the tumour, or from patients without skin tumours.[3,12,14] In this study, we examined the density and morphology of LCs in epidermal sheets of BCCs, perilesional skin and control skin from a large number of patients ($n = 65$).

To investigate the relationship between UV-exposed skin, LCs and BCCs, primary tumours from chronically sun-exposed skin (face) and more protected skin (trunk) were compared. To evaluate whether different staining procedures could explain the variations in results in previous reports, we used two different markers (OKT6 and ATPase) in a parallel fashion to identify LCs. These markers were co-expressed in BCC and in non-tumour-bearing skin in our study. The density of LCs was significantly decreased in the epidermal component of BCCs of both the face and trunk, compared with perilesional and control skin. There was no difference in LC counts between nodular and superficial BCCs. The ATPase$^+$ or OKT6$^+$ cells were sparsely distributed throughout the tumour-bearing epidermal sheets, but occasionally there were clusters of cells. This alteration of LC number was associated with morphological changes in the LC population in tumour specimens. Most cells were rounded and darkly stained with no dendrites, or short, stubby dendrites. Occasionally cells with a single, elongated dendrite without branches were found. Alterations in LC morphology have been noted in BCC and squamous cell carcinoma in other studies.[7,11,17] Some authors have proposed a regulating mechanism between LCs and keratinocyte growth and differentia-

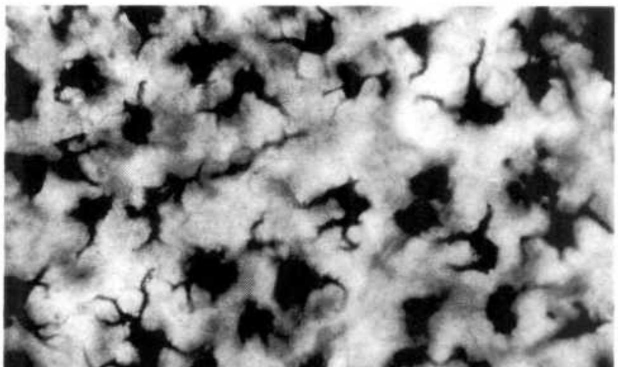

Figure 1. (a) Dendritic Langerhans cells in the epidermis in normal skin are demonstrated by ATPase staining. (b) Rounded Langerhans cells with no dendrites, or short dendrites, are demonstrated in the epidermis overlying the tumour (ATPase staining).

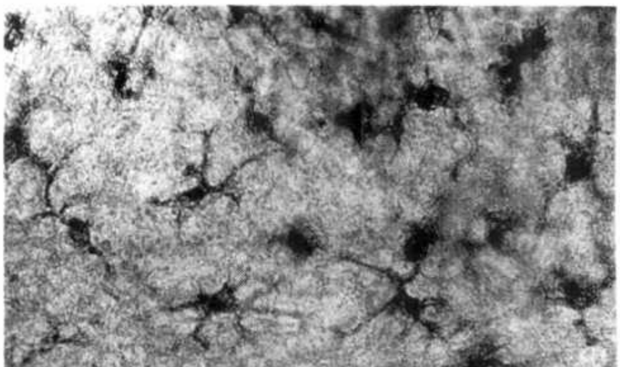

Figure 2. (a) Langerhans cells in normal skin (OKT6 staining). (b) Langerhans cells with elongated dendrites in the epidermal component of BCC (OKT6 staining).

tion.[11,18,19] This mechanism might be disturbed in BCC.

Whether BCC arises in areas deficient in LCs, or whether this reduction in LCs is a secondary phenomenon due to tumour growth, cannot be determined from this study. However, the alterations in LCs were confined to epidermis overlying tumours. LCs in perilesional skin of the face or trunk did not differ in density or morphology from LCs in control skin at the same location.

We therefore suggest that alterations in the number and morphology of epidermal LCs occur as a secondary event to tumour growth, either as a response to local factors produced by the tumour itself or as a non-specific reaction to changes in keratinocytes overlying BCCs.[7,17]

Acknowledgments

The authors wish to thank Tommy Johnsson for statistical analysis. This work was supported by grants from Edvard Welanders stiftelse.

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British Journal of Dermatology (1992) 127, 580–584.

A novel tissue preservation medium for immunoperoxidase staining of skin biopsies

A.CARMICHAEL, G.R.COGHILL, I.A.CREE, S.GEORGE* AND J.G.LOWE*

*Departments of Pathology and *Dermatology, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.*

Accepted for publication 12 May 1992

Summary

The requirement for unfixed tissue is a major drawback in the use of immunohistochemistry for the diagnosis of inflammatory and neoplastic disease. We describe the use of a novel gel transport medium (U.K. Patent No. WO 90/07703, international patent applications pending) to preserve unfixed skin biopsies from allergic contact dermatitis reactions for 1 week prior to frozen section and immunohistochemistry for leucocyte antigens. The medium can be used with biopsies up to 6 mm^3 and does not require any alteration in the usual frozen section or immunohistochemical staining procedures. The results show a subjective improvement in both morphology and staining quality. We believe this to be due to improved cutting of the sections and reduced background staining of collagen. This transport medium should be of considerable benefit to the provision of a clinical service, because it allows tissue for immunohistochemical examination to be taken at locations distant from the pathology laboratory.

Tissue samples for diagnosis are usually transported to the laboratory for histological examination in a fixative, the most popular of which is buffered formalin (4% formaldehyde). This simple method allows thin paraffin-embedded tissue sections to be prepared for routine histology. However, fixed tissue is often unsuitable for the demonstration of antigens by immunohistochemistry, due to the cross-linking effects of the fixative on proteins.[1] Many laboratories therefore ask for specimens requiring immunohistochemistry to be sent to them without fixation, to allow a fresh block to be taken for subsequent frozen section.

Frozen sections allow the demonstration of formalin-sensitive antigens, but the tissue must be frozen as soon as possible after it is obtained to preserve the antigens. This often means that the patient must attend the hospital nearest to the laboratory, necessitating an additional out-patient appointment. Alternatively, the tissue can be frozen at the outlying hospital or clinic and transported to the main laboratory in liquid nitrogen or carbon dioxode ice; this usually requires special arrangements to be made. Both methods can be inconvenient, and increase the cost of providing a diagnostic immunohistochemistry service.

For some years, we have been aware of the need for a fixative-free method of transporting tissue to the laboratory from outlying clinics or hospitals, and in the past, transport media such as Michel's transport medium, 5% glycerol, and Histocon have been used, with varying degrees of success.[2] We have recently developed a tissue transport medium capable of preserving cell membrane antigens, which allows the transport of tissue specimens at +4°C. To test the efficacy of the medium, we have examined a series of skin biopsies from patients with allergic contact dermatitis, to compare the conventional fresh biopsy and immediate frozen-section method with tissue kept in the transport medium for 1 week before frozen section and staining.

Methods

Biopsies

Punch biopsies (4 mm) were taken from 22 patients with allergic contact dermatitis reactions attending a dermatology out-patient department. Each biopsy was bisected immediately, using a razor blade. One half was frozen at once on metal foil, using liquid nitrogen, and sent to the laboratory. On receipt, the biopsy was stored in liquid nitrogen for 1 week. The other half of the biopsy was placed in the transport medium and sent to the laboratory, where it was kept in a refrigerator (0–4°C) for 1

Correspondence: Dr I.A.Cree, Department of Pathology, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.

week before it was mounted on a chuck and frozen using liquid nitrogen. Both biopsies were then sectioned at 6 μm in a Slee cryostat (Histological Equipment Ltd, Nottingham, U.K.).

Carmichael's transport medium

The medium consists of a sterile solution containing gelatin and cacodylate buffer which forms a gel at 4–8°C, but can be easily liquified when the container is warmed with warm water.[3] The biopsy is then fully immersed in the medium, and the specimen is stored at +4°C until required for frozen section. Carmichael's medium has recently become available commercially through Raymond A Lamb Ltd (London, U.K.).

Immunoperoxidase staining

Sections from both halves of each biopsy were stained simultaneously using an avidin–biotin complex (ABC) method (Vector Laboratories Ltd, Peterborough, U.K.), with a battery of monoclonal antibodies against inflammatory cell antigens (Table 1). The sections were counterstained with Mayer's haemalum, dehydrated, and mounted in DPX.

Histological assessment

The slides were assessed subjectively on a scale of 1 (excellent) to 5 (unreadable), for morphology and staining (Table 2), by two observers. The slides were compared side by side by the first observer (I.A.C.), and were randomized for grading by the second observer (J.G.L.).

Results

The first observer found the morphology and staining quality were better in the transport medium sections than in conventional frozen sections. The second observer, who graded randomized biopsies, confirmed the improvement in staining, but found no difference in morphology. The average grades given by both observers for each monoclonal antibody used are shown in Table 3.

Table 1. Battery of monoclonal antibodies employed for immunoperoxidase staining

Name	Company	Antigenic site
CD3	Dako	Pan-T-cell marker
CD4	Dako	Helper T-cell subset
CD8	Dako	Suppressor/cytotoxic T-cell subset
CD1	Dako	Langerhans cells
CD19	Dako	B cells
Mo	Dako	Macrophages
PC	Dako	Proliferating cell antigen
MC2 (CD15)	—	Neutrophils, fucosyl galactosamines

Table 2. Scale for assessment of staining and morphology

Grade	Description
1	Excellent
2	Good
3	Average
4	Poor
5	Unreadable

There was some difference, particularly of staining quality, between monoclonal antibodies, but most sections which had been in transport medium showed low background collagen staining, with normal or slightly reduced specific staining intensity. This was probably responsible for the better signal-to-noise, reflected in the better staining grade given to sections from transport medium-treated biopsies by both observers. To check that similar numbers of cells were being identified in skin biopsies, the number of stained cells present in halves of one skin biopsy, either snap frozen or stored in transport medium for 3 days, were assessed by counting within defined areas.[3–5] The results (Table 4) show no difference with CD4 antibody, but some increase in the number of cells stained with CD3 and CD8 antibodies in transport medium sections. This is thought to be due to improved staining in the transport medium-stored half of the biopsy (Fig. 1), although there may have been some difference in cell density between the two halves of the biopsy.

The subjective improvement in morphology found by the first observer on comparison of paired slides (Fig. 2) was probably due to reduced cutting artefact. Those who were cutting the sections invariably remarked upon the relative ease with which acceptable sections could be obtained from transport medium-treated tissue. Certainly, overall, there were fewer unusable or poor sections from transport medium-treated tissue. The time taken to orientate the biopsy on the chuck prior to freezing can be extended in comparison with fresh tissue. A proportion of the biopsies were taken from severe contact dermatitis reactions with incipient blistering. This was clearly visible in transport medium-treated biopsies, but not in the conventionally treated biopsies.

Table 3. Differences for all 22 biopsies in average staining quality and morphological grade for each monoclonal antibody used with frozen- or transport medium (TM)-treated tissue (1, excellent; 5, unusable)

Monoclonal antibody	Mean staining grade		Mean morphological grade	
	Frozen	TM	Frozen	TM
Observer 1 (direct comparison)				
CD3	1·73	1·18	2·50	1·41
CD4	2·68	1·82	2·86	1·73
CD8	2·14	1·68	2·82	1·82
CD1	2·09	1·32	2·86	1·73
CD19	2·36	1·50	2·91	1·77
Macrophage	2·59	1·27	3·36	2·00
Dako PC	2·36	1·68	3·23	1·95
MC2 (CD15)	2·64	1·73	3·27	1·91
All antibodies	2·32	1·52	2·97	1·79
Observer 2 (randomized grading)				
CD3	2·24	2·05	2·76	2·40
CD4	2·95	2 52	2·91	2 68
CD8	2·23	2·57	2·77	2·81
CD1	2·45	2·26	2·77	3 00
CD19	3·00	2·50	2·73	2·86
Macrophage	2·68	2·45	2·82	2·77
Dako PC	2·50	2·25	2·50	2·86
MC2 (CD15)	2·43	2·56	2·77	2·86
All antibodies	2·56	2·40	2·75	2·78

Table 4. Comparison of cell numbers per unit area counted in halves of a single skin biopsy stored in transport medium (TM) or frozen immediately

Monoclonal antibody	Clonal density (No./mm^3)	
	Frozen	TM
CD3	119·2	176·2
CD4	98·2	98·8
CD8	87·7	100·9

Likewise, spongiotic vesicles were easily seen in transport medium-treated biopsies (Fig. 2). Loss of the epidermis is a problem with frozen sections of skin biopsies. The prevalence of this problem was found to be similar in both transport medium and fresh-frozen biopsies.

Discussion

Previous attempts to produce a transport medium for fresh tissue have had limited success. Glycerol stabilizes the tissues and immobilizes the molecular structure, preserving antigenicity, but there is some loss of morphological detail.[6] Histocon, containing chlorhexidine and cacodylate buffer, is thought to have antibacterial and preserving properties which allow combined histology, enzyme histochemistry, and electron microscopy.[7] Michel *et al.*[8] used a similar approach to produce a transport medium for skin immunofluorescence, and this medium has subsequently been shown to be useful for preserving lymphocyte surface markers.[9] While most of these approaches allow limited tissue preservation for short periods, none has proved sufficiently robust to allow transport or storage of specimens over several days, and on occasion they have produced misleading results.[2]

In preliminary studies which led to the development of Carmichael's medium,[3] tonsillar tissue was preserved in a variety of different media, including glycerol, Histocon, and Michel's medium for up to 1 week before sectioning. The results with these media were disappointing in comparison with fresh-frozen sections and Carmichael's medium. The inclusion of an arsenical, sodium cacodylate, in our medium may cause some concern, but this compound has been used safely in similar quantities as a buffer for electron microscopy for many years.

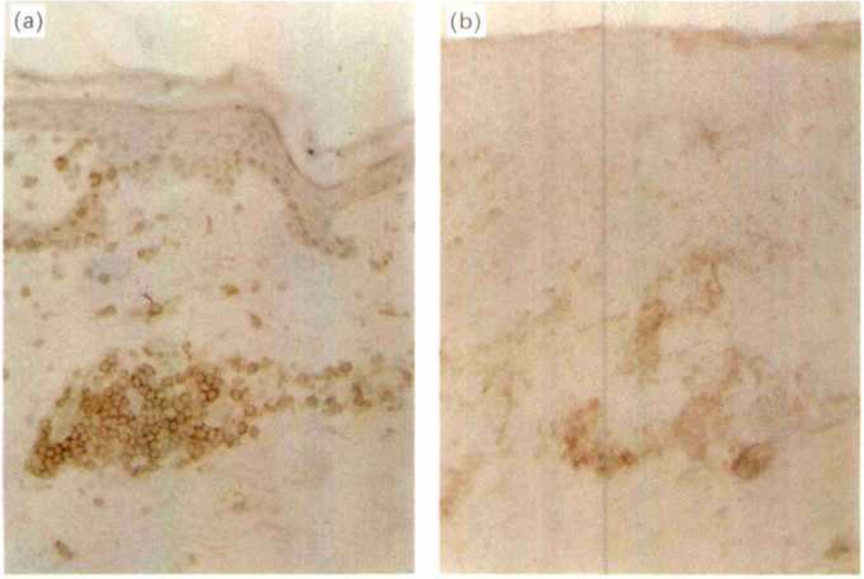

Figure 1. A high-power view of a skin biopsy, (a) half stored in transport medium and (b) half snap-frozen, both halves of which were cut and stained 1 week later with anti-CD4 antibody, showing improved cell localization in the thinner transport medium-treated section (× 350).

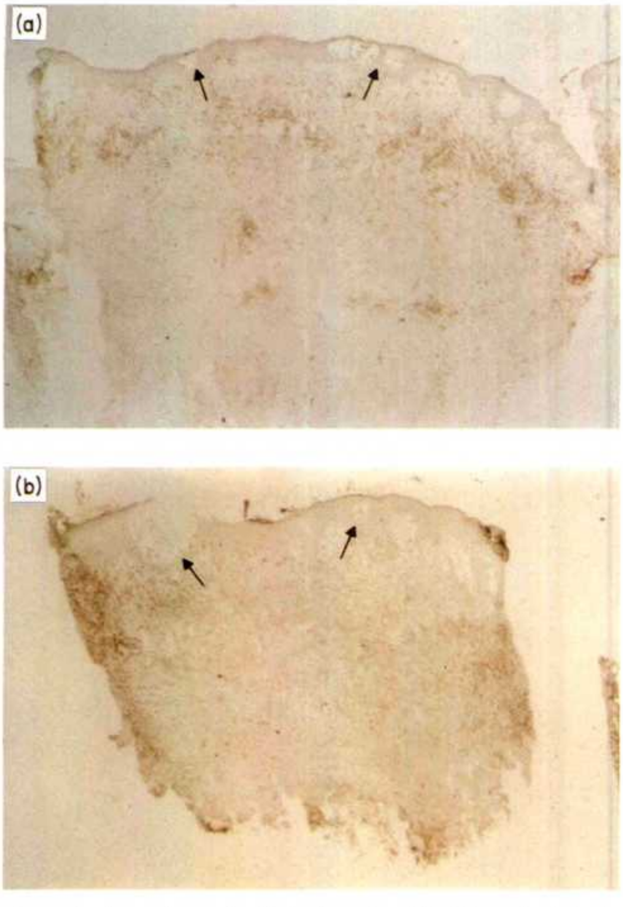

Figure 2. Comparison of both halves of a skin biopsy kept in (a) transport medium and (b) snap-frozen, showing improvement in morphology and staining with anti-CD3 immunostaining in the transport medium-treated tissue. Spongiotic vesicles are more clearly visible in the half of the biopsy stored in transport medium (arrows) (× 35).

The current study shows the superiority of transport medium over conventional treatment of tissue for the preparation of frozen sections. After 1 week's storage at 0–4°C the tissue staining and morphology is at least as good as that obtained in biopsies frozen immediately. These results can be obtained with biopsies taken at a considerable distance from the laboratory and transported to the main hospital before being processed. This avoids patients having to travel long distances to special biopsy clinics in the main centre, and means that biopsies can be performed at the time of the first visit. The improvement in quality is sufficient to permit image analysis of immunoperoxidase-stained sections, something we have previously found difficult with conventional frozen sections.

In the current study, we have examined the effect of storage for 1 week on skin biopsies. Recently, lymph-node biopsies have also been stained after storage in Carmichael's medium, and have produced equally impressive results (Cree *et al.* unpublished data), as have biopsies of oral mucosa stained for cytokeratins.[10] The medium has also been used to transport skin biopsies from Nepalese leprosy patients to this country, and to send gastrointestinal biopsies to a European laboratory. In these studies, no appreciable difference from previous frozen section studies was noted. Some of the Nepalese skin biopsies were kept for 3 weeks in transport medium before frozen sections were cut, and although there was some deterioration in the quality of staining, the results were still usable. Preliminary use of the medium in the immunofluorescence detection of immunoglobulin deposits in skin biopsies has been encouraging. There is an apparent decrease in background collagen staining. In one instance this resulted in the detection of IgA deposition at the dermal–epidermal junction which was not seen in the frozen section. However, we are concerned that complement C3 deposition may be less stable, and at present would suggest that biopsies intended for immunofluorescence are not stored in the medium for more than 24 h.

Morphological detail tends to be poorer in frozen sections compared with paraffin sections, so that often a choice has to be made of immunohistochemistry or morphology, but not both. We believe that this transport medium offers considerable advantages to pathology laboratories for the transport of small tissue specimens. Further testing of other tissue and antibody combinations will be required to determine how widely it can be applied.

Acknowledgments

This work was supported by Dundee Unitech Ltd. We also wish to thank Mr S.Macpherson and Mr R.Fawkes for photographic assistance.

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British Journal of Dermatology (1992) 127, 585–588.

Interleukin 1 immunoreactivity in sebaceous glands

H.S.I.ANTTILA, S.REITAMO AND J-H.SAURAT*
Department of Dermatology, Helsinki University Central Hospital, Finland
**Clinique de Dermatologie, Hôpital Cantonal Universitaire de Genève, Switzerland*

Accepted for publication 5 May 1992

Summary Interleukin 1 (IL-1) immunoreactivity in sebaceous glands was studied in paraffin sections of normal human skin. A panel of antibodies against IL-1α and β was tested using an immunoperoxidase labelling method. All the antibodies showed a similar specific labelling pattern: both glandular and ductal cells were immunoreactive for both IL-1α and β, provided optimal tissue fixation was used.

Interleukin 1 (IL-1) is present in normal human skin.[1] The major form of IL-1 in human skin *in vivo* is IL-1α.[2] IL-1β is present to a minor extent, although not in a biologically active form.[3] Several types of epidermal cells are capable of IL-1 synthesis, i.e. keratinocytes,[4] Langerhans cells[5] and melanocytes.[6] Dermal cells capable of IL-1 synthesis include fibroblasts,[7] mast cells, and trafficking cells such as those of monocyte–macrophage cell lineage.[8] A further source of IL-1 in the skin are the eccrine sweat glands, and both IL-1α and β can be detected in these by immunohistochemistry.[9] However, the major form of IL-1 in human sweat is IL-1α.[10]

In the present study we examined the distribution of IL-1 in normal human sebaceous glands using immunohistochemical methods.

Methods

Skin biopsies and tissue processing

Punch biopsies (2 or 3 mm) were obtained from the skin on the back of six male and three female volunteers, and the forearm of one male volunteer (ages 25–56 years). The biopsies were fixed either in Bouin's fluid, 10% formaldehyde in phosphate-buffered saline (PBS), 2·5% aqueous glutaraldehyde or Karnowsky fixative at 20°C for 90 min, and then routinely processed with alcohol and xylene to provide 4-μm paraffin sections.

In preliminary studies cryostat sections were also tested, but because of the poor preservation of the morphology of sebaceous glands in these sections, all further studies were performed with paraffin sections. Details of differences in IL-1 immunoreactivity related to different tissue-processing methods have been reported previously.[9–11]

Antibodies

A panel of antibodies against IL-1α and β was used in order to avoid the problem of unwanted reactions. They are listed in Table 1, and are similar to those used in our previous studies.[9,11] The effect of the fixation methods listed above on immunoreactivity was studied using only one antiserum (Cistron rabbit 02-1100). All further studies with other antibodies were performed using only Bouin's fluid as fixative, because in preliminary studies the other fixatives gave either poor preservation of immunoreactivity or non-specific labelling of dermal connective tissue (see Results for details).

Immunoperoxidase labelling

The immunoperoxidase labelling was performed using the ABC method for MAb and rabbit antisera, and the PAP method for sheep and goat antisera (Table 1). The sections were incubated with the respective antibodies for 30 min–3 h at room temperature, incubated in the dark with 0·025% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St Louis, MO, U.S.A.) and 0·003% H_2O_2 in phosphate-buffered saline (PBS), and finally counterstained with haematoxylin. After dehydration with alcohol and clearing with xylene the sections were mounted in Permount® (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

Controls

Three controls of immunolabelling were performed. (i) The primary anti-IL-1 antibody or antiserum was replaced by PBS or a non-relevant monoclonal antibody: anti-Leu 3a (Becton Dickinson, Mountain View, CA, U.S.A.), OKT6 (Ortho Diagnostic System, Raritan, NJ,

Table 1. Anti-interleukin 1 antibodies used for immunohistochemistry

Antibody	Species/isotype	Source*	Dilution	Immunoperoxidase method
Anti-interleukin 1β				
Cistron rabbit 02-1100	Rabbit	Cistron	1:50–1:200	ABC‡
Cistron MAb† 02-1025	Mouse IgM	Cistron	1:36	ABC
G MAb 7	Mouse IgG2a	Glaxo	1:350	ABC
G MAb 8	Mouse IgG2a	Glaxo	1:350	ABC
Sheep S77	Sheep	Glaxo	1:50	PAP§
Anti-interleukin 1α				
Sheep S76	Sheep	Glaxo	1:50	PAP
Goat G203	Goat	Glaxo	1:50	PAP

* Cistron Technology, Pine Brook, NJ, U.S.A.; Glaxo Institute of Molecular Biology, Geneva, Switzerland.

† MAb, monoclonal antibody.

‡ ABC, avidin–biotin complex, Vectastain ABC kit (rabbit IgG PK- 4001; mouse IgG PK-4002), Vector Laboratories, Burlingame, CA, U.S.A.

§ PAP, peroxidase–goat anti-peroxidase complexes, Dakopatts, Copenhagen, Denmark.

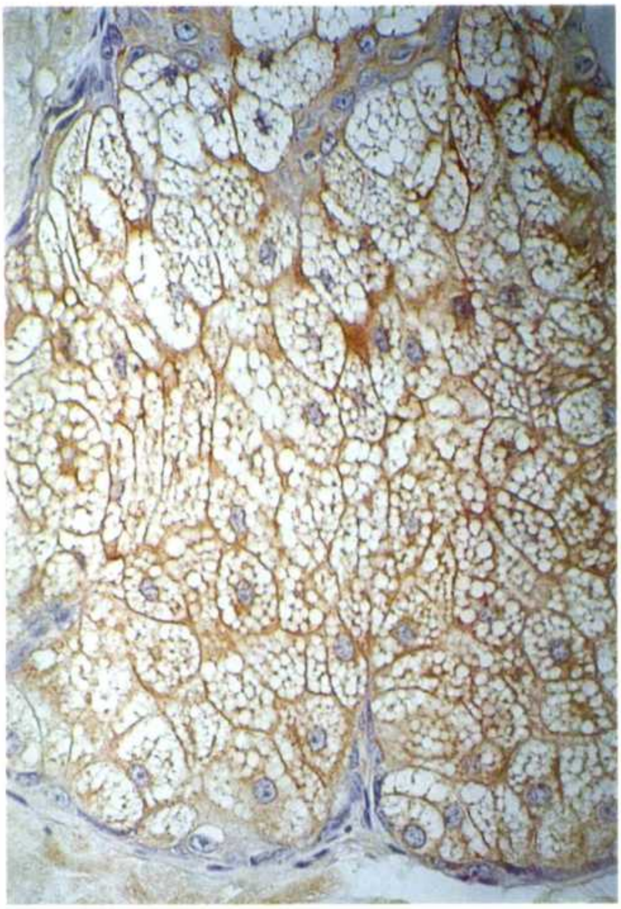

Figure 1. Bouin-fixed paraffin section from normal human skin labelled with a monoclonal antibody to IL-1β (G MAb 8). All sebaceous cells are immunoreactive (×400).

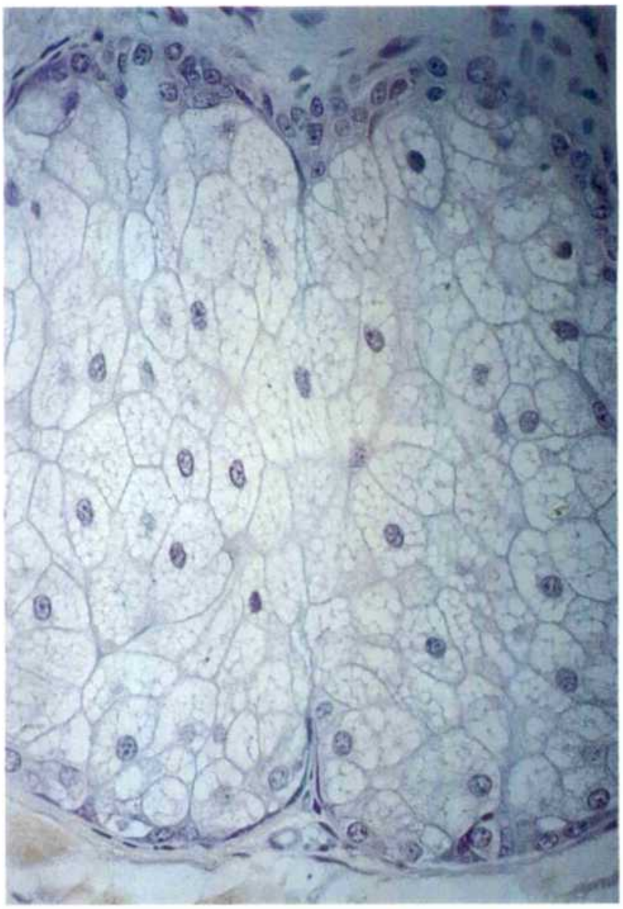

Figure 2. Parallel section to Figure 1. The immunolabelling was prevented by preabsorption of G MAb 8 by recombinant IL-1β.

U.S.A.), HLA-DR (Dakopatts, Copenhagen, Denmark). (ii) Tissue sections were stained for endogenous peroxidase activity without antibodies. (iii) For specific absorption 1 mg of human recombinant IL-1β was coupled to 100 μl of Sepharose beads as previously reported.[9,11] Monoclonal antibodies to IL-1β (G MAb 8) diluted 1:200 in Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) and 0·5% Nonidet P40 (LKB-Produkter, Bromma, Sweden) were absorbed on the beads at 37°C for 1 h following overnight incubation at 4°C, and again at 37°C for 1 h under agitation; for control of the procedure, diluted antibody was similarly processed without human recombinant IL-1. The absorbed and the non-absorbed antibodies were centrifuged at 10,000 *g* for 30 min at 4°C prior to immunoperoxidase labelling.

Results

Interleukin 1 immunoreactivity in sebaceous glands

All the monoclonal antibodies and polyclonal antisera showed a similar labelling pattern in the paraffin-embedded sections. All sebaceous cells were immunoreactive for various anti-interleukin 1 antibodies used (Table 2). A typical immunolabelling pattern is seen in Figure 1. No differences in the immunoreactivity between sebaceous glands from the back and forearm, or between the two sexes were seen.

Immunolabelling was prevented by absorption of monoclonal antibodies to IL-1β using human recombinant IL-1 prior to immunolabelling (Fig. 2). Other controls described in the Methods were negative. Table 3 shows the effect of the fixative on the immunolabelling with anti-IL-1 antibodies. In sections fixed in glutaraldehyde, sebaceous glands were immunoreactive for IL-1 antibodies, but an additional non-specific staining of dermal connective tissue was observed. Sebaceous glands fixed in formaldehyde or Karnowsky fixative showed no immunoreactivity for IL-1.

Table 2. Interleukin 1 immunoreactivity of human sebaceous glands fixed in Bouin's fluid

	Intensity of immunolabelling*		
	Cells of sebaceous gland lobi		
	Peripheral	Central	Ductal cells
Anti-interleukin 1α			
Sheep S76	+	+++	+++
Goat G203	−	++	+++
Anti-interleukin 1β			
Cistron rabbit 02-1100	+	++	++
Cistron MAb 02-1025	−	++	++
G MAb 7	+	+	++
G MAb 8	+	++	+
Sheep S77	+	+	++

* Graded from − to +++.

Table 3. Effect of fixation on the IL-1β immunoreactivity of human sebaceous glands*

	Intensity of immunolabelling		
	Cells of sebaceous gland lobi		
Fixation	Peripheral	Central	Ductal cells
Bouin	+	++	++
Glutaraldehyde	+	++	++
Formaldehyde	−	−	−
Karnowsky	−	−	−

* Immunolabelling with Cistron rabbit 02-1100, intensity graded from − to +++.

Discussion

We have shown by immunohistochemical methods that both interleukin 1α and β can be detected in normal human sebaceous glands. It is of interest that all the cell layers of the sebaceous gland acini were immunoreactive for IL-1α and β. Our previous studies also identified immunoreactivity in all the cell layers of the human epidermis,[11] when appropriate fixation procedures were used. Earlier studies[12] had suggested that IL-1 immunoreactivity was limited either to the basal cells or the stratum granulosum. Removal of lipids from the sebaceous glands by the tissue-processing methods did not alter IL-1 immunoreactivity, indicating that it is not associated with sebaceous lipids. This is of interest because IL-1 can be recovered from the skin surface. It has previously been shown that IL-1 present in sweat accounts for this finding. Moreover, the highest amount of surface IL-1 was recovered from the palms and soles, where there are no sebaceous glands. Taken together these findings suggest that IL-1 might not be as important a component of excreted sebum as of excreted sweat. This remains, however, to be directly evaluated.

A recent study suggested that inflammatory levels of IL-1α-like material are present in the majority of open comedones in acne vulgaris.[13] Levels of IL-1β were much lower, which is in accordance with previous studies showing that IL-1α is the major IL-1 of human skin and sweat.[2,10] It has been shown that IL-1α purified from normal human skin, and injected intradermally, causes rapid influx of neutrophils into the skin. This effect could be mediated through interleukin 8 which can be induced by IL-1.[14] It is therefore probable that sebaceous gland IL-1 participates in the inflammatory events in the dermis following sebaceous gland disruption. To what extent this is related to the pathogenesis of acne vulgaris remains to be established. It is notable that topical retinoic acid was found not to modulate the epidermal content of IL-1.[15] To our knowledge, the effect of systemic isotretinoin on sebaceous gland IL-1 has not been evaluated. Recent findings suggest that interleukin 1 levels in comedones are increased after treatment of acne with tetracycline.[16]

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British Journal of Dermatology (1992) 127, 589–594.

Adenylate cyclase induces intracellular calcium increase in single human epidermal keratinocytes measured by fluorescence microscopy using Fura 2-AM

C.YASUI, H.KOIZUMI, T.FUKAYA, M.KUMAKIRI, A.OHKAWARA AND T.UEDA*

Department of Dermatology, Hokkaido University School of Medicine, Sapporo, Japan
**Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan*

Accepted for publication 1 April 1992

Summary

Intracellular calcium ($[Ca^{2+}]i$) is an important second messenger of extracellular signals to induce various cellular responses. Extracellular and intracellular Ca^{2+} are considered to be important for cellular differentiation and proliferation of epidermal keratinocytes. Several mechanisms which increase $[Ca^{2+}]i$ have been demonstrated in various tissues, but in epidermal keratinocytes these mechanisms are poorly understood. In epidermal keratinocytes the adenylate cyclase–cyclic AMP response is thought to regulate cell proliferation and differentiation. However, the series of reactions which follow the cyclic AMP response remain unknown. Beta-adrenergic agonists increase $[Ca^{2+}]i$ in cultured epidermal keratinocytes, and we have therefore studied whether stimulation of keratinocyte adenylate cyclase could induce $[Ca^{2+}]i$ increase, by using fluorescence microscopy with Fura 2-AM.

Adenosine and histamine, which are known to be keratinocyte adenylate cyclase receptor agonists, induced transient $[Ca^{2+}]i$ increase, as did epinephrine. In addition, forskolin, a direct adenylate cyclase activator, and dibutylyl-cyclic AMP also induced an increase in $[Ca^{2+}]i$. In a calcium-free medium epinephrine, adenosine, histamine and dibutylyl-cyclic AMP induced an increase in $[Ca^{2+}]i$. These results suggest that cyclic AMP in human epidermal keratinocytes regulates $[Ca^{2+}]i$, which is released from intracellular stores.

Keratinocytes in the basal layer proliferate and differentiate to form the stratum corneum and maintain the constant thickness of vital epidermal cell layers. Investigation of their regulatory mechanisms has disclosed many signal-transducing mechanisms in the skin. Intracellular free calcium ($[Ca^{2+}]i$) is regarded as one of the most important factors in these mechanisms. Many calcium-dependent cellular responses have been demonstrated to have significance in keratinocytes,[1,2] but the mechanisms of changes in $[Ca^{2+}]i$ are unclear.

Beta-adrenergic agonists induce an increase in $[Ca^{2+}]i$ in cultured normal human keratinocytes, as previously described,[3] and they are one of the stimuli which activate the adenylate cyclase–cyclic AMP system in the epidermis.[4] We have therefore investigated modulation of the $[Ca^{2+}]i$ of single cultured monolayer human keratinocytes by stimuli which activate the epidermal adenylate cyclase-cyclic AMP system, using fluorescence microscopy employing a highly fluorescent Ca^{2+} sensitive dye, Fura 2-AM,[5] as previously described.[3,6,7]

Methods

Chemicals

All chemicals were freshly prepared for each experiment, and the pH of the medium was adjusted to 7·4. Epinephrine was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan), forskolin from Carbiochem-Behring Corp. (La Jolla, CA, U.S.A.), Fura 2-AM from Sigma (St Louis, MO, U.S.A.), and other chemicals from Wako Pure Chemical Co. (Osaka, Japan).

Culture of normal human keratinocytes

Human neonatal foreskin keratinocytes were cultured in serum-free medium according to the method of Boyce and Ham.[8] Human epidermal keratinocytes grow rapidly in biochemically defined KGM culture medium consisting of basal medium MCDB153 supplemented

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Correspondence: Dr H.Koizumi, Department of Dermatology, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo, 060 Japan.

with 0·5 μg/ml of hydrocortisone, 5 ng/ml of epidermal growth factor, 5 μg/ml of insulin, and 0·5% bovine pituitary extract, in an atmosphere of 95% air and 5% CO_2 at 37°C. The Ca^{2+} concentration of the medium was 0·15 mM. Cells in third passage were used for the experiments. Keratinocytes of the proliferative phase with 60–80% confluence were used. Twenty-four hours before the experiments the culture medium was changed to KBM medium in which growth factors and hormones were depleted.

Measurement of intracellular free calcium

The changes in $[Ca^{2+}]$i in single keratinocytes were measured by fluorescence microscopy using Fura 2-AM, as previously described.[3,6] The keratinocytes were loaded with 5 μM of Fura 2-AM for 1 h at 37°C in the dark. After washing, the cells in the KBM medium were placed in a chamber maintained at 37°C by regulating two thermomodules. The chamber was placed on the stage of an inverted fluorescence microscope. The excitation wavelength was varied between 340 and 380 nm by moving respective interference filters to and fro through a solenoid. The fluorescence image was monitored by a SIT videocassette (Ikegami Co., model CTC-900), and transmitted to a microcomputer (NEC, model PC-9801VM) through a videodigitizer (Attec System Company, model ALT256-8), which digitized a frame into 256 × 256 points with 256 brightness levels. The ratio of the fluorescence at 340 nm to that at 380 nm, at each point in the cells, was calculated. Compounds dissolved in the KBM medium were added to the cell chamber for the stimulation experiments. In each experiment five points in different cells were monitored. The modulation of $[Ca^{2+}]$i was analysed at each point. The experiments were repeated using more than three different dishes. The addition of the KBM medium to the cell chamber did not cause any alteration of fluorescence ratio at 340 and 380 nm. The relationship between the Fura-2 fluorescence ratio and concentration of Ca^{2+} was studied in a cell-free system as follows. The fluorescence of a series of 10 μM of Fura-2-AM-containing solutions buffered with 1 mM EGTA and various concentrations of $CaCl_2$ to precise Ca^{2+} from 20 to 500 nM was measured as described above. The relationship between the Fura-2-AM fluorescence ratio (340/380 nm) and free calcium increased linearly, corresponding to linear changes in $\log^{10}$ concentration from 20 to 500 nM. The estimated $[Ca^{2+}]$i of cultured epidermal keratinocytes from the calibration curve was 45 ± 10 nM at a steady state. It is not concluded that this is an actual concentration because the fluorescence is affected by proteins in the cells, or kDa of Fura-2-AM, in the living cells. However, this concentration was at a similar level to that reported in other cells, and therefore the estimated concentration may be assumed to be near to the intracellular Ca^{2+} concentration. The modulation of $[Ca^{2+}]$i in living single cells could be measured by this method.

Results

Cultured human neonatal foreskin keratinocytes took Fura 2-AM into the cells and showed fluorescence (Fig. 1). Control keratinocytes without Fura 2-AM loading showed no fluorescence (data not shown). The ratio of the fluorescence at 340 nm to that at 380 nm of individual cells of the cultured keratinocytes was recorded. Perfusion of the KBM medium into the chamber did not affect the fluorescence of the cells. The calcium ionophore (2 μM), A23187, induced an immediate increase in the ratio (data not shown). A23187-induced calcium influx into the cytoplasm of the keratinocytes was detected.

Time courses of $[Ca^{2+}]$i in response to adenosine (0·1–10 mM) and histamine (0·01–10 mM), the receptor agonists of adenylate cyclase of human epidermis, were investigated. Adenosine, 1–10 mM, transiently increased $[Ca^{2+}]$i (Fig. 2a). 0·1–10 mM of histamine also increased $[Ca^{2+}]$i in the keratinocytes (Fig. 2b) with a slower recovery to the initial level. Forskolin, which is a direct activator of adenylate cyclase,[9] induced immediate and transient $[Ca^{2+}]$i increase at 1 mM (Fig. 3a). A similar $[Ca^{2+}]$i increase was also observed when the

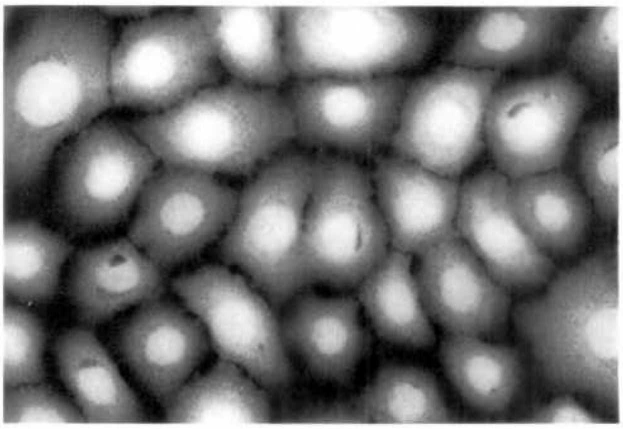

Figure 1. Fura 2-AM loaded cultured human epidermal keratinocytes observed with inverted fluorescence microscope. Keratinocytes were incubated in serum-free KBM medium with 5 μM of Fura 2-AM at 37°C for 1 h.

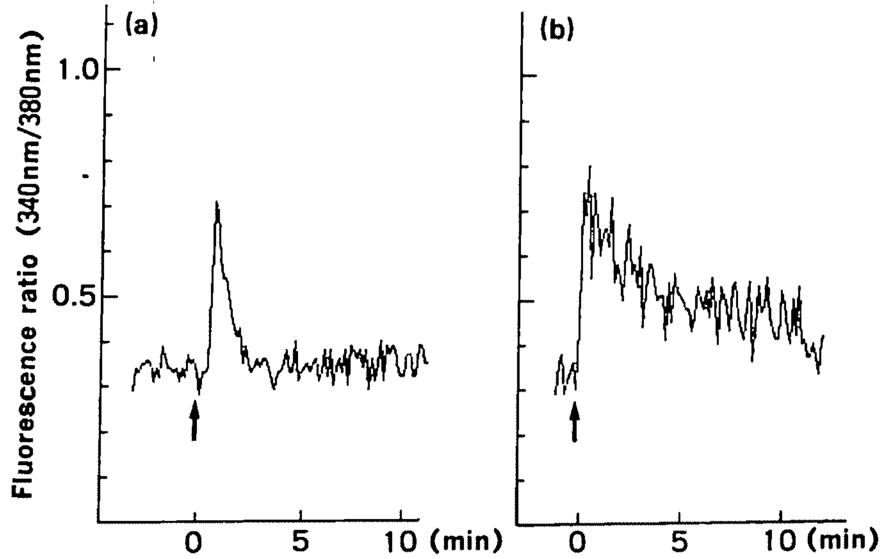

Figure 2. [Ca^{2+}]i increase in keratinocytes induced by histamine and adenosine. (a) Adenosine (1 mM) and (b) 0·1 mM of histamine were perfused on to the chamber, at the times shown by the arrows.

keratinocytes were stimulated with 10 mM of dibutylyl-cyclic AMP (Fig. 3b). The [Ca^{2+}]i elevation induced by adenosine, histamine, and forskolin is in accord with our results on human pure epidermal sheets,[10] and the magnitude of calcium fluxes was similar in the epidermal sheets and in cultured monolayer keratinocytes.

When the cells were pre-incubated with 2 mM EGTA, epinephrine, adenosine, histamine, or dibutylyl-cyclic AMP could induce an increase in [Ca^{2+}]i (Fig. 4; Table 1). These results demonstrate that the Ca^{2+} was released from an intracellular store in keratinocytes.

With two stimulations by 50 μM of epinephrine in 3 min, a transient [Ca^{2+}]i increase was observed only on the first addition of epinephrine (data not shown). However, with a double stimulation of the cells by 50 μM of epinephrine and 1 mM of adenosine in 3 min, keratinocytes showed a [Ca^{2+}]i increase after each stimulation (Fig. 5). These results show that the elevation of [Ca^{2+}]i was due to the respective receptor-mediated mechanism.

Discussion

In this study, receptor-mediated calcium mobilization was demonstrated in cultured human epidermal keratinocytes. This is operated through adenylate cyclase

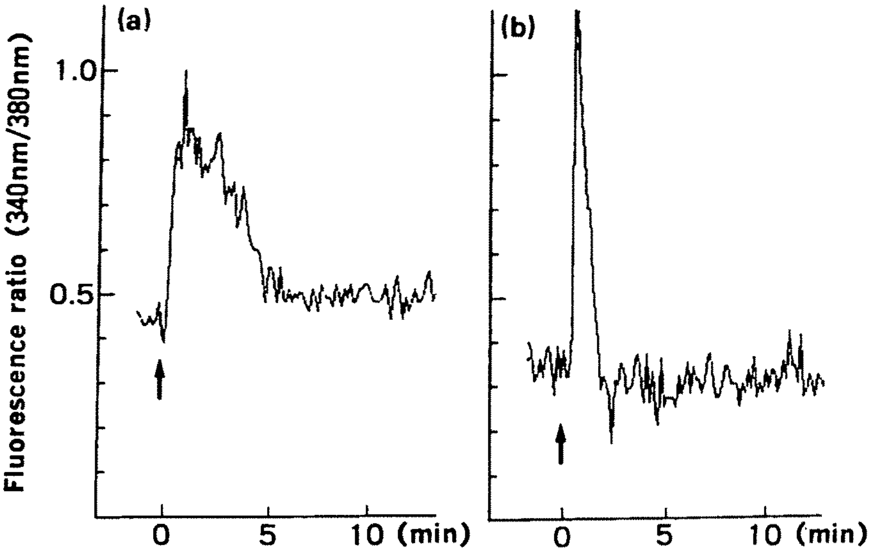

Figure 3. Effects of forskolin and dibutylyl-cyclic AMP on [Ca^{2+}]i. (a) Forskolin (1 mM), and (b) 10 mM of dibutylyl-cyclic AMP were perfused on to the chamber at the times shown by the arrows.

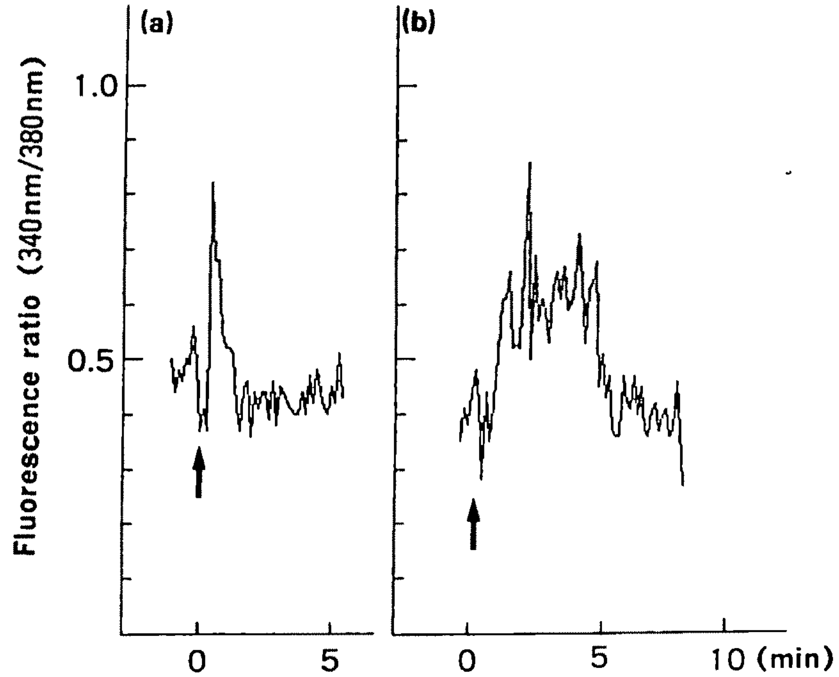

Figure 4. Effects of extracellular calcium on $[Ca^{2+}]i$ increase induced by epinephrine and dibutylyl-cyclic AMP. The keratinocytes were incubated in KBM medium in which calcium was chelated with 2 mM EGTA.
(a) Epinephrine (50 µM) and
(b) 10 mM of dibutylyl-cyclic AMP were added at the times shown by the arrows.

Table 1. Effects of the stimuli on $[Ca^{2+}]i$

Agents	$[Ca^{2+}]i$ increase Calcium in medium +	 −
Receptor agonists		
Epinephrine (50 µM)	+	+
Histamine (1 mM)	+	+
Adenosine (1 mM)	+	+
Adenylate cyclase activator		
Forskolin (1 mM)	+	ND
Cyclic AMP derivative		
Dibutylyl-cyclic AMP (10 mM)	+	+

ND, not determined.

activation, resulting in the release of Ca^{2+} from an intracellular store.

The adenylate cyclase system in human epidermal keratinocytes is known to have at least four receptors for beta-adrenergic adrenalin, histamine, adenosine and prostaglandin E, based on stimulation studies and immunohistochemical studies.[4] Binding of agonists to the receptors induces activation of adenylate cyclase through stimulatory GTP binding protein, and adenylate cyclase produces cyclic AMP from ATP. In human

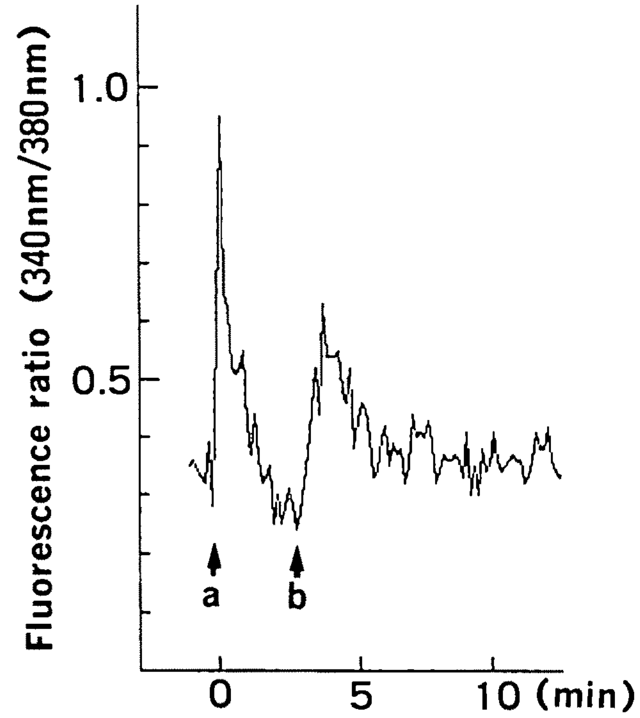

Figure 5. Effects of successive stimulation by epinephrine and adenosine on $[Ca^{2+}]i$. Epinephrine (50 µM) was perfused on to the chamber at the time shown by the arrow (a). Three minutes later the cells were stimulated by 1 mM of adenosine shown by arrow (b).

keratinocytes the prostaglandin E–adenylate cyclase response is weak.

Beta-adrenalin, histamine, adenosine, and cyclic AMP derivative were shown to increase [Ca^{2+}]i in cultured human epidermal keratinocytes, and forskolin, which is a direct stimulator of adenylate cyclase, also induced an increase in [Ca^{2+}]i. The effective concentrations of these compounds are similar to those which activate adenylate cyclase. These stimulants also increase [Ca^{2+}]i in human pure epidermal sheets, as previously reported.[10] [Ca^{2+}]i is concerned with many calcium-dependent cellular responses such as calmodulin-dependent protein kinase activation, desmosome formation, and cellular differentiation.[4,11] The exact relation of these cellular responses to changes of [Ca^{2+}]i remains to be clarified. In psoriatic epidermis, alteration of adenylate cyclase responsiveness has been reported.[4] Drugs used in the treatment of psoriasis augment adenylate cyclase responses *in vitro*.[12] Adenylate cyclase is regarded as one of the key enzymes for epidermal keratinocyte proliferation and differentiation. Therefore, some of the regulatory effects of adenylate cyclase on epidermal cell kinetics are assumed to involve [Ca^{2+}]i mobilization. It is reported that agents which raise cyclic AMP levels increase [Ca^{2+}]i in human T lymphocytes, smooth muscle, and other cells,[13–16] as well as in human keratinocytes, as shown in this study. As with adenylate cyclase, the mechanisms inducing [Ca^{2+}]i increase have not been fully elucidated. GTP binding protein directly regulates the calcium channel in cardiac muscle.[17] Cyclic nucleotides directly activate ion channels, for example cyclic GMP in photoreceptors and cyclic AMP in olfactory receptors.[18] It has been suggested that in skeletal and cardiac muscle cells, cyclic AMP-dependent phosphorylation of calcium channel protein increases open-state probability of the channel.[13–15] Phosphorylation of inositol-1,4,5-trisphosphate (IP3) receptor by cyclic AMP-dependent protein kinase decreases its release of calcium in rat brain.[19] However, the significance of this phosphorylation remains to be clarified.[20]

The [Ca^{2+}]i mobilization mechanisms in epidermal keratinocytes are not limited to the adenylate cyclase–cyclic AMP system. Phospholipase C and protein kinase C have been demonstrated in keratinocytes, and these enzymes play important roles in keratinocyte regulation.[21–24] In this transmembrane signalling system, IP3, which is produced by phospholipase C, induced [Ca^{2+}]i release from an intracellular store.[25] Changes of [Ca^{2+}]i by stimulation with phospholipase C and protein kinase C are under investigation in our laboratory. The mechanism of [Ca^{2+}]i mobilization is complex and may be interfered with by other transmembrane signalling systems. The detailed mechanisms of adenylate cyclase-mediated increase of [Ca^{2+}]i and the subsequent calcium-dependent cellular reactions are also under investigation.

Acknowledgement

This work was supported in part by a Grant-in-aid for Scientific Research No. 02670469 awarded to H.Koizumi from the Ministry of Education.

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British Journal of Dermatology (1992) 127, 595–599.

Substance P induces intracellular calcium increase and translocation of protein kinase C in epidermis

H.KOIZUMI, H.TANAKA, T.FUKAYA AND A.OHKAWARA
Department of Dermatology, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kita-Ku, Sapporo, 060, Japan

Accepted for publication 30 April 1992

Summary

Substance P is a neuropeptide present in, and released from, peripheral C nerve endings. The presence of substance P-positive nerve fibres in the epidermis has been reported. We investigated the effect of substance P on the transmembrane signalling system of pig epidermal keratinocytes. Treatment of pig epidermis with substance P resulted in an increase in inositol 1,4,5-trisphosphate (IP_3), and in intracellular free calcium. The treatment also resulted in translocation of protein kinase C from a cytosol to a membrane fraction. Substance P, however, did not affect the β-adrenergic- or histamine (H2)- adenylate cyclase responses of the epidermis. Neither forskolin-induced, nor cholera toxin-induced cyclic AMP accumulation were affected by substance P treatment. These results are consistent with the view that substance P stimulates phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis of keratinocytes, resulting in IP_3-Ca^{2+} and diacylglycerol-protein kinase C signal activation. Although protein kinase C is known to affect the epidermal adenylate cyclase system, no evidence for such 'cross-talk regulation' was detected in keratinocytes by substance P treatment.

Substance P is a neuropeptide, which is present in unmyelinated nerve endings in the skin.[1,2] Substance P-positive nerve endings have been demonstrated in the epidermis and dermis of normal and psoriatic skin, and psoriatic plaques are more densely innervated than normal skin.[3] The content of substance P is also reported to be elevated in psoriatic skin.[4]

Substance P is released from peripheral nerve endings by various stimuli. Farber *et al.*[5] suggested that substance P might have pathophysiological significance in psoriasis. According to their view, substance P released from the peripheral nerve endings affects various cell systems directly or indirectly, resulting in the development of psoriatic lesions. For example, substance P induces histamine release from mast cells, which results in cutaneous vasodilatation,[6] and Tanaka *et al.*[7] reported that substance P increases Pam 212 keratinocyte proliferation. Although the latter finding has not been confirmed unequivocally by studies using normal human keratinocytes in culture,[8,9] these findings could be related to the formation of the erythematous, hyperproliferative lesions in psoriasis.

There is little additional information about other biological aspects of the effects of substance P on keratinocytes. Interestingly, substance P has been shown to stimulate phosphatidylinositol turnover and to activate protein kinase C in other cell systems.[10,11] The part played by these signalling systems in epidermal proliferation and differentiation, which are deranged in various pathological conditions of the epidermis, including psoriasis,[12,13] is a matter of interest. Consequently, we investigated the effect of substance P on various transmembrane signalling systems in epidermal keratinocytes.

Methods

Chemicals

Substance P, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), phosphatidylserine, and cholera toxin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). (γ-^{32}P)ATP and D-myo-inositol 1,4,5-trisphosphate (IP_3) [^{3}H] assay system were purchased from Amersham Japan Corp. (Tokyo, Japan). Spantide was purchased from Bachem Inc. (CA, U.S.A.). Forskolin was obtained from Calbiochem Co. (La Jolla, CA, U.S.A.). Cyclic AMP assay kit is a product of Yamasa Shoyu Co. (Tokyo, Japan).

Protein kinase C preparation

Skin slices were obtained from the backs of domestic pigs (weight 5–10 kg) with a Castroviejo keratome set at 0·2

Correspondence: Dr H.Koizumi.

mm thickness. The skin slices were treated with 1000 U/ml of dispase in RPMI 1640 medium for 30 min at 37°C. Following the dispase treatment, the pure epidermal sheets were peeled off with sharp forceps. The epidermal sheets were then floated on fresh RPMI 1640 medium for 30 min at 37°C, and were incubated with 50 μM of substance P for 15 min. Following incubation, the epidermal sheets were wiped with filter paper, and immediately homogenized in a conical glass homogenizer with 5 vol (w/v) of 20 mM Tris HCl, pH 7·5, containing 0·25 M sucrose, 2 mM EDTA, 0·5 mM EGTA, and 2 mM phenylmethylsulphonyl fluoride (PMSF). The enzyme source was centrifuged at 105,000 *g* for 60 min. The supernatant and the pellet were used as the source of protein kinase C in the cytosol fraction and in the membrane fraction, respectively. These crude extracts were applied to a DE52-cellulose column and the enzyme was eluted with 1·5 column volumes of 0·08 M NaCl. Protein kinase C activity was measured as previously described.[14]

Detection of intracellular calcium alteration

Alteration of intracellular Ca^{2+} of single living epidermal keratinocytes was measured by the method of Williams *et al.*[15] using a fluorescent Ca^{2+}-sensitive dye, Fura 2-AM and inverted fluorescence microscopy, as previously described.[16] Pure epidermal sheets were pre-incubated for 30 min, and then incubated with Fura 2-AM for 30 min. The keratinocytes were placed on the stage of an inverted fluorescence microscope. Fifty micromoles of substance P and 1 mM of histamine were added to the cells in succession. Five points of different cells were recorded simultaneously.

Measurement of inositol 1,4,5-trisphosphate

Slices of epidermal sheets (5 × 10 mm) were pre-incubated in RPMI 1640 medium for 20 min at 37°C in a water bath, and then incubated in fresh medium with 50 μM of substance P. The tissues were rapidly frozen on dry ice after 10–120 s incubation.[17] Otherwise the epidermal sheets were incubated with different concentrations of substance P for 30 s. The samples were then homogenized with 4 N perchloric acid and kept on ice for 20 min. Proteins were sedimented by centrifugation at 2000 *g* for 15 min at 4°C. Supernatants were titrated to pH 7·5 with 7·5 mM HEPES, 1·54 mM KOH, and kept on ice for 30 min. Precipitated $KClO_4$ was sedimented and removed. The supernatant was used as samples for IP_3. IP_3 content in the samples was measured by a radiochemical method using a D-myo-inositol 1,4,5-trisphosphate assay system.

Cyclic AMP accumulation

Pig epidermal slices obtained by dispase treatment were cut into pieces 5 mm² with a razor blade, and were rinsed three times in RPMI 1640 medium. They were then floated on the buffer with the keratin layer uppermost. The epidermal slices were pre-incubated at 37°C for 20 min in RPMI 1640 medium in the water bath.

For receptor-stimulated cyclic AMP accumulation, the specimens were incubated at 37°C for 5 min in the RPMI 1640 medium with 50 μM epinephrine or 0·1 mM histamine, in the presence or absence of 50 μM of substance P. The slices were quickly frozen between two plates of dry ice to terminate the reaction, and cyclic AMP content was determined. Both epinephrine and histamine are agonists of the receptors for adenylate cyclase responses.

In other experiments, epidermal slices were incubated with 100 μg/ml of cholera toxin and 1 mM IBMX for 3 h, or with 100 μM forskolin and 1 mM IBMX for 1 h, in the

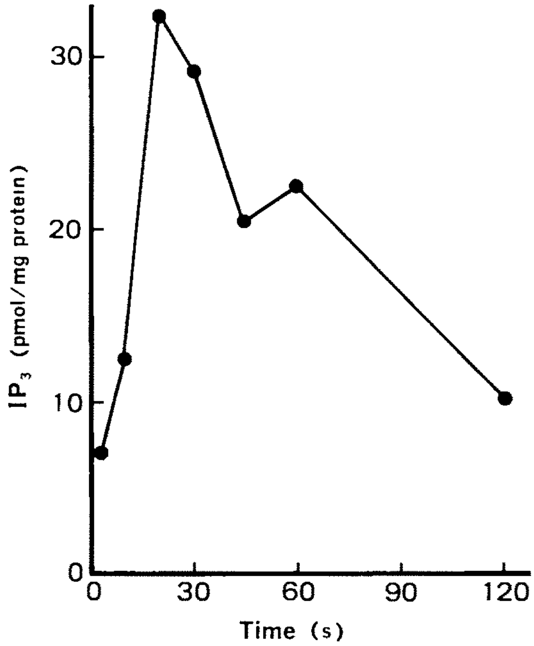

Figure 1. The time course of the effect of substance P on inositol 1,4,5-trisphosphate accumulation in pig epidermis. Pig epidermal sheets were incubated in RPMI 1640 medium with 50 μM of substance P at 37°C in a water-bath. The IP_3 contents were measured as described in the text. Data are expressed as means of triplicate samples. Essentially identical results were obtained by three different experiments.

Table 1. Effects of spantide on inositol 1,4,5-trisphosphate accumulation by substance P

	Inositol 1,4,5-trisphosphate (pmol/mg protein)	
Control	14·3±2·8	
		$P<0{\cdot}025$
50 μM substance P	31·0±3·7	
		$P<0{\cdot}025$
50 μM spantide + 50 μM substance P	17·9±1·4	

Data are expressed as means±1 SE of quadruple samples. The difference between control and spantide+substance P levels is not statistically significant.

presence or absence of 50 μM of substance P. The incubation was terminated as described above. Forskolin is a direct activator of adenylate cyclase. Cholera toxin causes accumulation of cyclic AMP through GPT binding protein.

Cyclic AMP content in these skin slices was measured by radioimmunoassay using a Yamasa cyclic AMP assay kit, as previously described.[18]

The protein content was determined using the method of Lowry *et al.*[19]

Results

Substance P produces IP_3 increase

Treatment of pig epidermal keratinocytes with 50 μM of substance P resulted in an IP_3 increase. The IP_3 formation was a rapid and transient process reaching its peak at 20 s, then gradually decreasing (Fig. 1). Pretreatment of the epidermis with 50 μM spantide for 5 min inhibited the effect of substance P on IP_3 accumulation ($P<0{\cdot}025$) (Table 1). Substance P-induced IP_3 accumulation was a dose-dependent process reaching a plateau level at 100 nM (Fig. 2). Thrombin is known to increase IP_3 in cultured human keratinocytes,[20] and treatment of pig epidermal keratinocytes with 0·1 IU/ml of thrombin for 20 s also resulted in an increase in IP_3 in our system (data not shown).

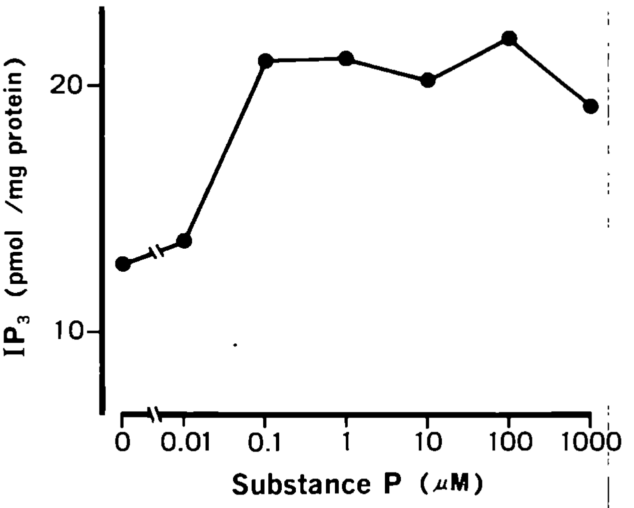

Figure 2. The concentration effect of substance P on inositol 1,4,5-trisphosphate accumulation in pig epidermis. Pig epidermal sheets were incubated in RPMI 1640 medium with the indicated concentration of substance P at 37°C for 30 s. IP3 content was measured as described in the text.

Substance P induces intracellular Ca^{2+} increase

Treatment of pig epidermal keratinocytes with 50 μM of substance P resulted in an immediate and transient increase in intracellular Ca^{2+} (Fig. 3). Histamine (1 mM) also induced an intracellular Ca^{2+} increase with a similar time course (Fig. 3). We have previously reported that histamine increases intracellular Ca^{2+} in human epidermal keratinocytes.[21]

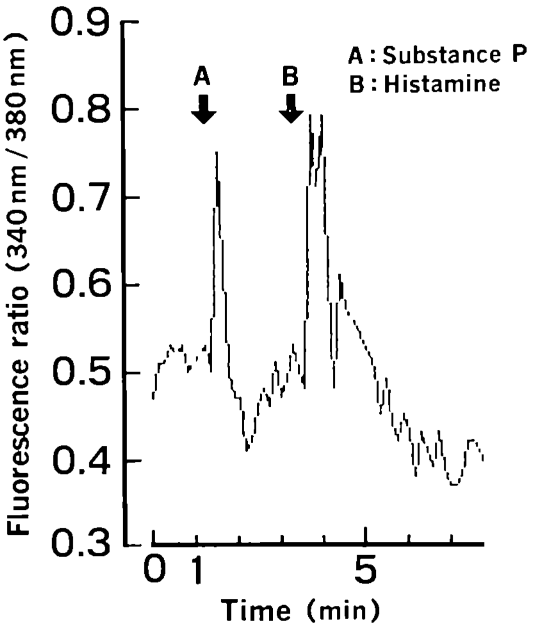

Figure 3. Alteration of intracellular Ca^{2+} by substance P and histamine in pig epidermal sheets. The alteration of intracellular Ca^{2+} of single living epidermal keratinocytes was measured using Fura 2-AM. Five points of different cells were recorded simultaneously, with similar results. Essentially identical results were obtained in two independent experiments.

Table 2. Effects of substance P on protein kinase C activity

	Protein kinase C activity (pmol/min/mg protein)	
	Cytosol	Membrane
Control	11·1±2·7	14·0±3·4
50 μM substance P	2·9±0·3*	23·3±2·1

Data are expressed as means±1 SE of triplicate samples. *$P<0{\cdot}05$ compared with the control group.

Substance P induces translocation of protein kinase C from a cytosol to a membrane fraction

Pig epidermal slices were incubated with 30 μM of substance P for 30 min. Treatment of the epidermis with substance P for 15 min induced translocation of protein kinase signal. No evidence for such 'cross-talk' regulation by substance P was obtained in our system (Table 3).

Effects of substance P on adenylate cyclase system

Substance P did not affect the receptor-adenylate cyclase responses of the epidermis; neither the β-adrenergic- nor the histamine (H2)-adenylate cyclase response of the epidermis was affected by substance P (Table 3). Forskolin is a direct activator of adenylate cyclase, and cholera toxin induces sustained stimulation of stimulatory guanine nucleotide binding protein. Neither forskolin-, nor cholera toxin-induced cyclic AMP accumulation was affected by substance P treatment (Table 3).

Table 3. Effects of substance P on the adenylate cyclase system of the epidermis

	Cyclic AMP (pmol/mg protein)	
	Control	Substance P
50 μM epinephrine	18·5±0·2	20·2±0·8
0·1 mM histamine	34·9±5·8	30·6±4·7
100 μM forskolin	121·7±11·9	123 4±6·6
100 μg/ml cholera toxin	98·2±1·9	102·7±6·4

Data are expressed as means±1 SE of triplicate samples. There is no statistically significant difference between control and substance P groups.

Discussion

Substance P increased IP$_3$ and intracellular Ca^{2+} of pig epidermal keratinocytes *in vitro*. Substance P also induced translocation of protein kinase C from a cytosol to a membrane fraction. Although we did not measure diacylglycerol formation, the latter finding is indirect evidence for the activation of protein kinase C. The results are consistent with the view that substance P stimulates phosphatidylinositol-4,5-bisphosphate (PIP$_2$) hydrolysis of keratinocytes, resulting in IP$_3$-Ca^{2+} and diacylglycerol-protein kinase C signal activation. Iizuka *et al.*[22,23] reported that pig epidermal adenylate cyclase responses are affected by the diacylglycerol-protein kinase signal. No evidence for such 'cross-talk' regulation by substance P was obtained in our system (Table 3).

Substance P has been shown to stimulate phosphatidylinositol turnover and activate protein kinase C in various cell systems. These transmembrane signalling systems play an important role in different cells. For example, substance P induces contractile responses in guinea-pig ileum and rabbit iris sphincter muscles.[10,11] Substance P also stimulates histamine release from skin mast cells,[6] induces expression of an endothelial–leucocyte adhesion molecule in microvascular endothelium,[24] and stimulates proliferation of fibroblasts, lymphocytes and macrophages.[25,26]

The lesions of psoriasis and prurigo nodularis are densely innervated by substance P-positive nerves.[3,27] Although the direct effect of substance P on proliferation and keratinization remains to be determined, we have shown for the first time that substance P affects the phosphatidylinositol metabolism of keratinocytes. Because the phosphatidylinositol breakdown system is one of the major transmembrane signalling systems of keratinocytes influencing proliferation and keratinization, the substance P-induced signalling system should have profound effects on keratinocyte biology. It is known that the phosphatidylinositol breakdown system is deranged in various pathological conditions of keratinocytes, including psoriasis.[28,29]

Investigations into the effect of substance P on keratinocyte proliferation and differentiation are under way in our laboratory, as are investigations into the effects on keratinocyte biology of other neuropeptides present in the skin.

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British Journal of Dermatology (1992) 127, 600–607.

Rat hair follicle growth *in vitro*

M.P.PHILPOTT, M.R.GREEN* AND T.KEALEY

Department of Clinical Biochemistry, University of Cambridge, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QR, U.K.
**Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.*

Accepted for publication 9 June 1992

Summary

Pelage hair follicles were isolated by gentle microdissection from 8–12-day-old rats, and maintained in supplemented Williams E medium. Length measurements made on freshly isolated hair follicles, and at 24-h intervals, showed a significant increase in hair follicle length over 48 h, after which time no further significant increase in length was observed. Photomicrographs of maintained follicles showed that this increase in hair follicle length could be attributed to the production of a keratinized hair shaft. Histology and [methyl-^{3}H] thymidine autoradiography of freshly isolated hair follicles showed the dermal papilla to be elongated, with thymidine uptake located predominantly in the matrix cells of the hair follicle bulb adjacent to the dermal papilla. This pattern remained unaltered for the first 48 h of maintenance, but after 72 h the dermal papilla had rounded into a tight ball of cells, with very little thymidine uptake occurring in the adjacent matrix cells.

On maintenance, fetal calf serum (FCS), epidermal growth factor (EGF) and 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) all significantly stimulated [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake, but inhibited hair follicle elongation. Insulin-like growth factor-1 (IGF-1) had no significant effect on rates of hair follicle elongation and [methyl-^{3}H] thymidine uptake, but significantly stimulated rates of [U-^{14}C] leucine uptake. Transforming growth factor-β1 (TGF-β1) significantly inhibited both the rate of [methyl-^{3}H] thymidine uptake and hair follicle elongation.

The *in vitro* growth of hair follicles over a 4–5 day period in skin plugs taken from post-embryonic mouse skin has been reported.[1] However, these studies did not permit detailed measurements to be made on individual hair follicles. Attempts at culturing individual hair follicles from post-embryonic mouse[2,3] and rat skin[4] have met with little success. We have shown that rat hair follicles isolated by 'shearing'[5] appear to be viable and intact on isolation, but when maintained *in vitro* on permeable supports fail to grow.[6] We recently reported the successful growth of isolated human hair follicles *in vitro*,[7] showing that by careful isolation methods it is possible to obtain undamaged human hair follicles which grow *in vitro* at an *in vivo* rate. This present study describes the *in vitro* growth of rat hair follicles isolated by an improved gentle microdissection technique. The effects of EGF, TGF-β1, IGF-1, and TPA on the rate of hair follicle elongation were examined, together with their effects on the rates of [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake and on hair follicle viability as assessed by their adenosine triphosphate (ATP) content.

Methods

Materials

Williams E medium, L-glutamine, Fungizone®, penicillin/streptomycin and colchicine were supplied by Gibco. All other tissue culture supplements and mouse EGF and TPA were from Sigma. All radiochemicals were supplied by Amersham; ATP monitoring kits were supplied by Pharmacia LKB. Porcine TGF-β1 was from R & D Systems Inc., and synthetic human IGF-1 was supplied by Bachem Feinchemikalien.

Isolation of rat hair follicles by microdissection

Rat hair follicles were isolated by a simple microdissection method. Strips of dorsal skin (2–5 × 0·5 cm) were removed from 8–12-day-old Wistar rats. These strips were cut into small pieces, approximately 0·5 cm^2, and individual pieces were placed in a Petri dish containing isolation medium (PBS/EBSS, 1:1). The skin was gently torn into small strips under a binocular microscope using watch-maker's forceps. Hair follicles were then isolated from the skin by gentle manipulation with the watch-maker's forceps. Isolated hair follicles were

Correspondence: Dr M.P.Philpott.

immediately transferred to experimental media as described below.

Hair follicle maintenance

(i) Elongation measurements. Individual, freshly isolated hair follicles were maintained at 37°C in an atmosphere of 5% CO_2/95% air in separate wells of 96-well plates containing 150 μl of Williams E medium with 1% FCS, 2 mM L-glutamine, insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone (10 ng/ml), sodium selenite (10 ng/ml), Fungizone® (2·5 μg/ml), penicillin/streptomycin (100 U/ml: 100 μg/ml) and trace element mix (Gibco). Measurements were made using a Nikon Diaphot inverted microscope equipped with an eyepiece measuring graticule.

(ii) Organ maintenance. Organ maintenance of freshly isolated rat hair follicles was carried out using four-well plates. Each well contained 4 ml of Williams E medium supplemented as described above. Hair follicles were maintained free-floating, with 20 follicles in each well, in an atmosphere of 5% CO_2/95% air, at 37°C.

Rates of DNA and protein synthesis

These were investigated by measuring the rates of incorporation of [methyl-^{3}H] thymidine and [U-^{14}C] leucine, respectively, into perchloric acid (PCA) precipitable material, as previously described.[7] Incubations were carried out in plastic Eppendorf tubes containing 500 μl of Williams E medium supplemented with 1 μCi of 3 μM [methyl-^{3}H] thymidine (specific activity 0·67 mCi/μM) and 0·5 μCi of 0·5 mM [U-^{14}C] leucine (specific activity 2 mCi/mM). Follicles in Eppendorf tubes were incubated in stoppered plastic tubes containing 0·5 ml of distilled water in a gently shaking water bath at 37°C in an atmosphere of 5% CO_2/95% O_2 for 3 h. Follicles were then washed three times with PBS supplemented with 10 mM thymidine and 10 mM leucine, and then homogenized in 1 ml of 0·1 M EDTA, pH 12·3. This was then centrifuged for 15 min at 12,000 *g* to precipitate cell debris, and the supernatant was removed for assay. Aliquots (100 μl) were removed for total DNA assay.[7] Macromolecules in the remaining supernatant were precipitated by the addition of 500 μl of 25% (v/v) PCA, and the resulting precipitate was collected on to Whatman GF/C filters, under vacuum. Filters were washed with 10 ml of 10% (w/v) trichloroacetic acid (TCA) followed by 5 ml of 5% (w/v) TCA, and dried with 1 ml of ethanol/diethyl ether (1:1). Radioactivity was counted in 10 ml of Optifluor® scintillant, using dual counting liquid scintillation spectrometry.

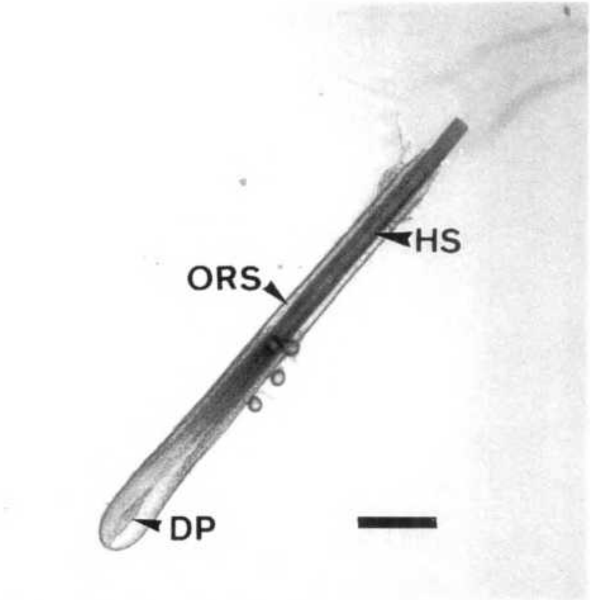

Figure 1. Light micrograph of a freshly isolated hair follicle from a 10-day-old rat showing hair follicle bulb with dermal papilla (DP), hair shaft (HS) and outer root sheath (ORS) (scale bar = 200 μm).

Autoradiography

This was carried out as described previously.[7] Briefly, hair follicles were incubated for 6 h with 5 μCi of [methyl-^{3}H] thymidine. Following incubation, the follicles were washed three times with PBS supplemented with 10 mM thymidine, and fixed for 1 h with 3%

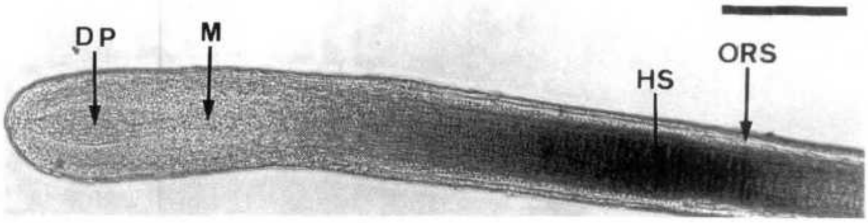

Figure 2. Light micrograph of the bulb region of an isolated rat hair follicle at higher power, showing the dermal papilla (DP), matrix (M), outer root sheath (ORS) and hair shaft (HS) (scale bar = 200 μm).

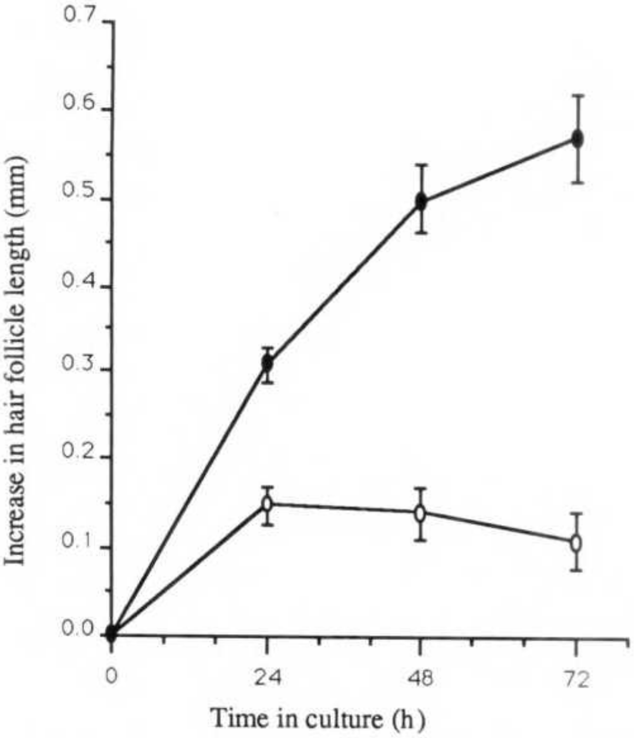

Figure 3. Changes in rat hair follicle length with time in culture. Experiments were carried out as described in the text. Results are expressed as the mean ± SEM for $n = 6$ rats (six follicles from each rat, 36 follicles in total). Statistical analysis of data was carried out using Student's *t*-test. ●, control; ○, colchicine.

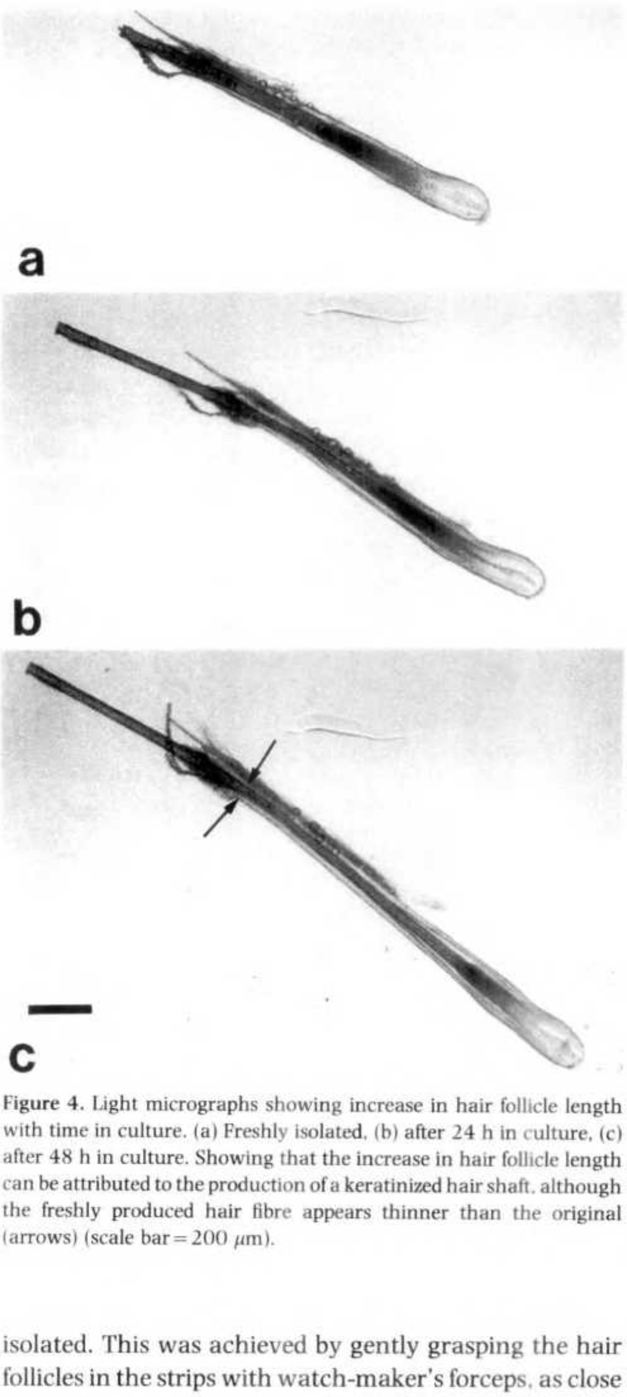

Figure 4. Light micrographs showing increase in hair follicle length with time in culture. (a) Freshly isolated, (b) after 24 h in culture, (c) after 48 h in culture. Showing that the increase in hair follicle length can be attributed to the production of a keratinized hair shaft, although the freshly produced hair fibre appears thinner than the original (arrows) (scale bar = 200 μm).

glutaraldehyde. Follicles were then mounted in 5% agar to assist handling during subsequent processing. The agar blocks were fixed overnight in 3% glutaraldehyde, then washed in PBS and processed into Historesin. Sections (1·5 μm) were cut, and autoradiography was carried out using K5 dipping emulsion (Ilford).

Hair follicle ATP content

This was measured using LKB-ATP monitoring kits based on the luciferin/luciferase assay of ATP.[8] Hair follicles were placed in 500 μl of Williams E medium, to which was added 100 μl of 20% PCA. Twenty microlitres of sample was removed and neutralized with 1 M KOH. Aliquots (10 μl) were made up to 90 μl with 0·1 M Tris-Acetate buffer, pH 7·75, and the ATP content measured using LKB-ATP monitoring reagent and a LKB 1250 luminometer.

Results

It was found that, by cutting rat skin into thin strips, large numbers of intact individual hair follicles could be isolated. This was achieved by gently grasping the hair follicles in the strips with watch-maker's forceps, as close to the dermal/subcutaneous fat interface as possible. By gently pulling downward, it was found that the follicles could easily be removed from the skin (Fig. 1). When observed under an inverted binocular microscope at higher power (Fig. 2), hair follicles showed an apparently intact morphology. When maintained free-floating in individual wells of 96-well plates, they showed a significant increase in length over 48 h in culture (0·24 mm/day, $P < 0·001$; Fig. 3). However, after 48 h in

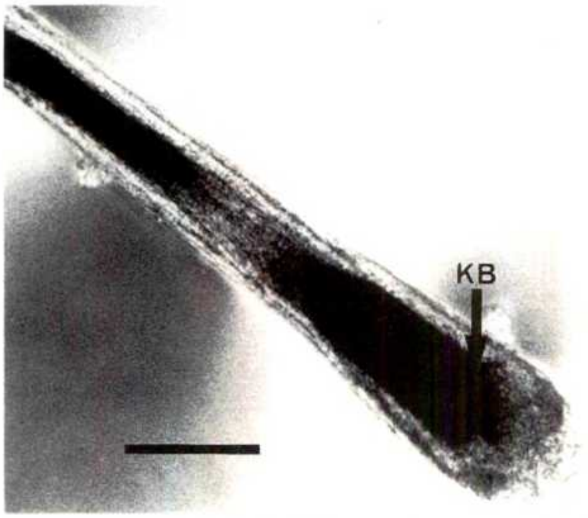

Figure 5. Light micrograph showing the apparent keratinization of the hair follicle bulb after 72 h in culture (scale bar = 200 μm).

culture, this rate of increase in length was reduced to 0·06 mm/day. Figure 3 also shows that, when hair follicles were incubated with colchicine (0·6 μg/ml), an inhibitor of mitosis, hair follicle growth was significantly inhibited. Figure 4 shows that the increase in hair follicle length with time in culture can be attributed to the production of a keratinized hair shaft. It was frequently observed that, after 48 h in culture, a wave of keratinization would apparently move downwards towards the base of the hair follicle, and usually resulted in the formation of a keratinized hair follicle bulb (Fig. 5).

[Methyl-^{3}H] thymidine autoradiography showed that in freshly isolated rat hair follicles (Fig. 6a) [methyl-^{3}H] thymidine uptake was located in the matrix cells of the hair follicle bulb. After 48 h in culture (Fig. 6b), the uptake was still located in the matrix cells of the hair follicle, and no significant change in hair follicle morphology was observed. After 72 h in culture, however (Fig. 6c), the dermal papilla had transformed from an elongated structure to a spherical cluster of cells at the base of the follicle, with very little thymidine uptake occurring in the adjacent matrix cells.

It was observed that increasing concentrations of FCS (Fig. 7) significantly inhibited rat hair follicle growth *in vitro*, as did TPA, EGF and TGF-β1 (Fig. 8). Paradoxically, FCS, TPA and EGF all significantly stimulated [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake (Table 1), but TGF-β1 significantly inhibited both [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake. IGF-1 had no significant effect on either hair follicle growth (Fig. 8) or [methyl-^{3}H] thymidine uptake, but did significantly stimulate [U-^{14}C] leucine uptake (Table 1). Control experiments, in which the ATP content of hair follicles was measured, showed that the growth inhibitory effects could not be attributed to cytotoxic cell damage (Table 1), although TPA was observed to raise ATP content significantly.

Discussion

Isolated rat hair follicles were maintained *in vitro* for up to 48 h, during which time they showed a significant increase in length. After 48 h in culture, the follicles showed no further statistically significant increase in length, and the matrix cells of the hair follicle appeared to keratinize, giving rise to a 'club-like' structure. Autoradiography showed that matrix cells of the hair follicle continued to incorporate [methyl-^{3}H] thymidine for at least 48 h after isolation, during which time the dermal papillae of the maintained follicles retained their typical elongated structure, as seen in freshly isolated follicles. After 72 h maintenance, the dermal papilla formed a spherical cluster of cells situated at the base of the hair follicle bulb, and labelling of matrix cells was considerably reduced, a feature typical of the onset of catagen, although some thymidine uptake was observed in the cells of the hair follicle outer root sheath (ORS). When we maintained isolated rat hair follicles with the anti-mitotic agent colchicine, which acts by arresting spindle formation and thus blocking the cell in metaphase, they showed no further increase in length after 24 h in culture, indicating that mitotic cell division underlies hair follicle elongation *in vitro*. These data are consistent with the conclusion that isolated rat hair follicles can show true hair growth *in vitro* for at least 48 h, and that these changes are not due to swelling or other artefactual changes. After 48 h in culture, it would appear that, although DNA synthesis in the matrix cells of the hair follicles stops, these cells continue to differentiate, giving rise to the formation of a keratinized hair follicle bulb.

It is not clear why rat hair follicles do not continue to grow for more than 48 h in culture, when human follicles can be maintained for at least 9 days.[9] The rat hair follicles do not appear to be damaged on isolation, as far as may be determined by light microscopy. However, rat hair follicles isolated from 8–12-day-old rats are much smaller than human hair follicles, and they may be exposed to greater trauma on isolation than human hair follicles. It is well known that when hair follicles are stressed they respond by premature entry into catagen.[10] Moreover, as the first hair growth cycle of the rat is very short, and these follicles would normally enter catagen

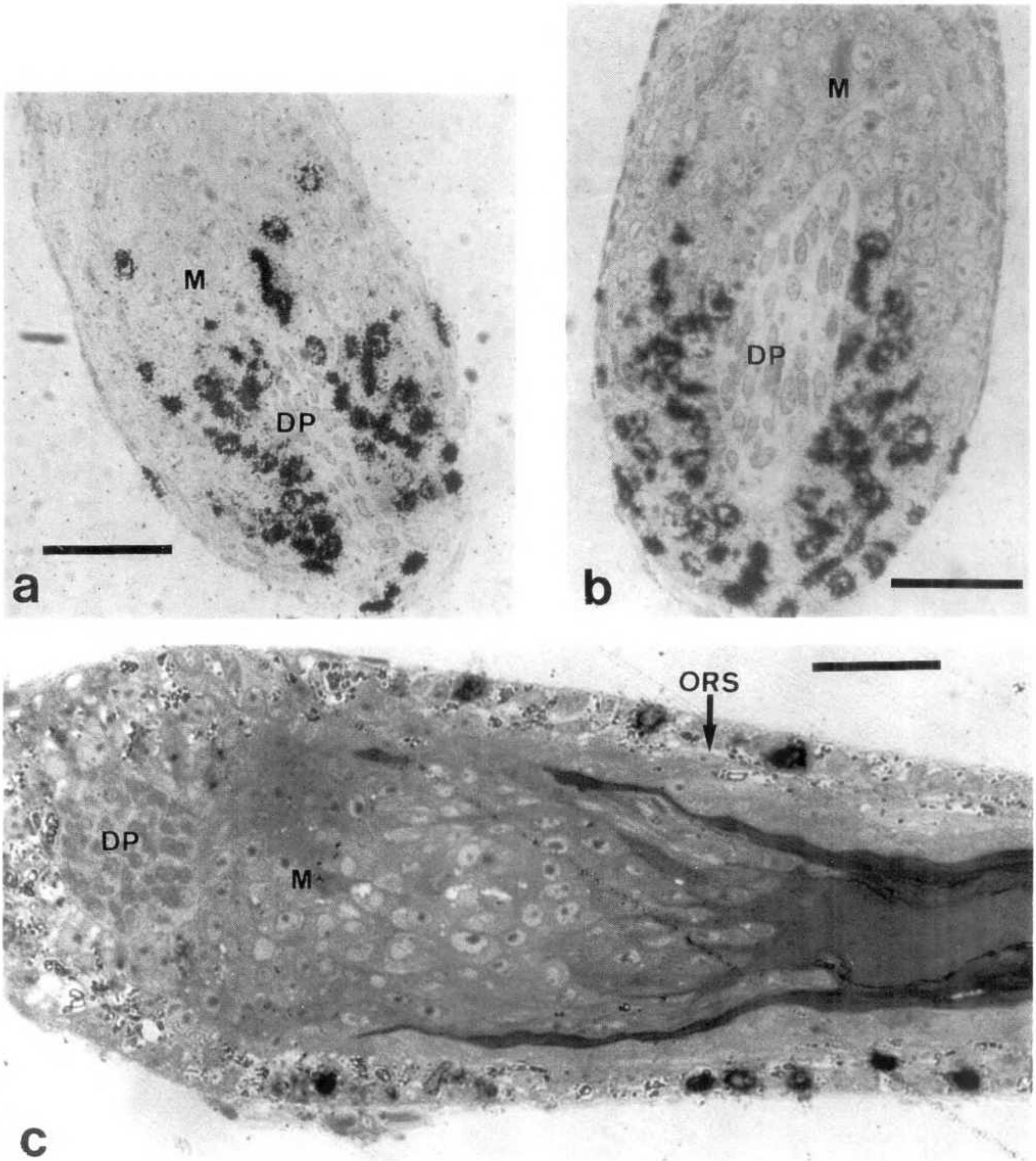

Figure 6. Autoradiography, showing the patterns of [methyl-^{3}H] thymidine uptake in: (a) freshly isolated rat hair follicles, with the majority of uptake in the matrix cell region of the hair follicle adjacent to the dermal papilla (DP); (b) after 48 h in culture the dermal papilla remains elongated, and the majority of uptake still occurs in the adjacent matrix cells; (c) after 72 h in culture considerable changes have taken place in the hair follicle bulb; the dermal papilla has formed a rounded structure, and very little uptake takes place in the adjacent matrix cells (scale bar = 100 μm).

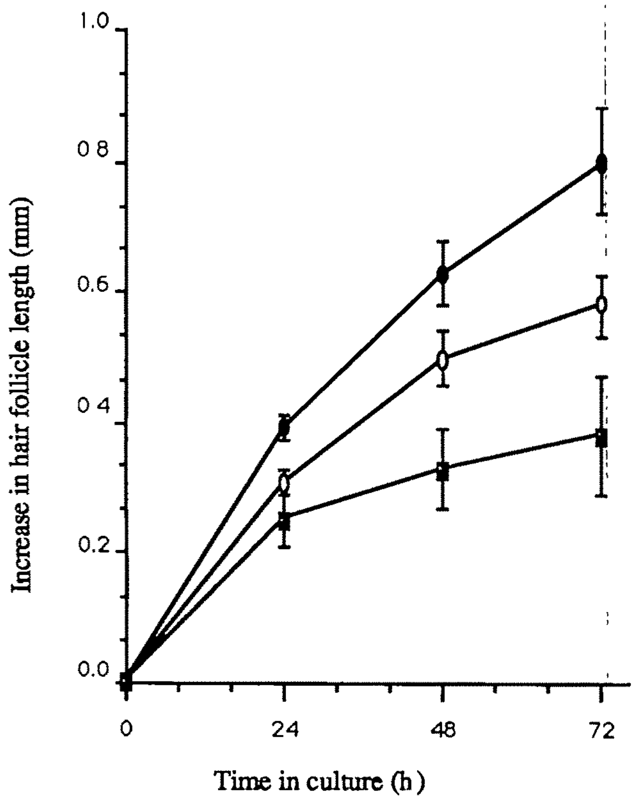

Figure 7. The effects of serum on the length of isolated rat hair follicles maintained *in vitro*. Experiments were carried out as described in the text. Results are expressed as the mean ± SEM for $n = 6$ rats (6 follicles from each rat, 36 follicles in total). Statistical analysis of data was carried out using Student's *t*-test. ●, serum free; ○ 1% FCS; ■, 20% FCS.

at around 17 days of age,[11] it is possible that as a result of the trauma of isolation they are more likely to enter a catagen-like state prematurely. It would therefore be interesting to discover whether hair follicles isolated from older rats in either the third or fourth hair growth cycle, in which the follicles have a more prolonged anagen phase, can be maintained for longer. It has also been shown that steroid hormones regulate the activity of rat pelage follicles,[12] and the sudden change in hormonal environment in culture may have more effect on rat pelage follicles than human scalp hair follicles.

Increasing concentrations of FCS were found to significantly stimulate both [methyl-^{3}H] thymidine uptake and [U-^{14}C] leucine uptake, but hair follicles grew significantly longer in serum-free medium than the follicles maintained in either 1 or 20% FCS; this is similar to our observations on isolated human hair follicles, in which we found that serum significantly inhibited hair follicle growth *in vitro*.[7,9]

It was observed that, when rat hair follicles were maintained *in vitro* with FCS, cells explanted from the ORS region of the hair follicle, whereas this did not occur in hair follicles maintained in serum-free medium. Thus, serum seems to contain factors that stimulate ORS cell division, but inhibit matrix cell division. These results suggest that in isolated rat hair follicles FCS is able to stimulate ORS cell division, but to specifically inhibit the matrix cells which are responsible for hair follicle growth. These factors have yet to be fully characterized, although TGF-β is known to be present.[13] In this study we have shown that, when freshly isolated rat hair follicles are maintained *in vitro* in the presence of 10 ng/ml TGF-β1, hair follicle elongation was significantly inhibited, but ATP content remained unchanged, suggesting that the observed inhibition of hair follicle growth was not due to a cytotoxic effect. Interestingly, TGF-β, which inhibits the growth of human epidermal keratinocytes *in vitro*, does not inhibit their protein synthesis over 3 days.[14] We observed the same uncoupling in the hair follicle, in that TGF-β inhibited [methyl-^{3}H] thymidine uptake but not [U-^{14}C] leucine uptake. These observations are similar to those we reported for isolated human hair follicles,[7] and indicate that in the hair follicle TGF-β1 may act as both a negative growth regulator and a promoter of hair follicle keratinocyte maturation.

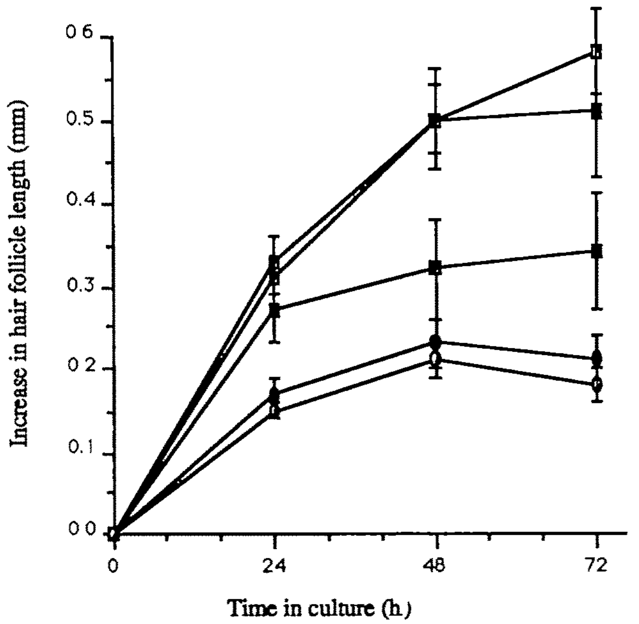

Figure 8. The effects of growth factors and mitogens on the length of isolated rat hair follicles maintained *in vitro*. Experiments were carried out as described in the text. Results are expressed as the mean ± SEM for $n = 6$ rats (36 follicles in total). Statistical analysis of data was carried out using Student's *t*-test. □, control; ■, IGF-1; ⊡, TGF-β1; ●, EGF, ○, TPA.

Table 1. The effects of growth factors and mitogens on the rates of [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake and hair follicle ATP content of isolated rat hair hair follicles maintained for 24 h. Numbers in parentheses indicate number rats used for each observation

Treatment	[methyl-^{3}H]thymidine pmol/μgDNA/3h	[U-^{14}C] leucine pmol/μgDNA/3h	Hair follicle ATP pmol/20 follicles
Serum free	1.29±0.21 (6)	196±29 (6)	291±36 (4)
1% FCS	1·22±0·14 (19)	156±15 (19)	250±27 (4)
10% FCS	1·94±0·32 (6)	319±107 (6)	Not measured
20% FCS	2·57±0·24††† (12)	310±44†† (12)	347±68 (4)
EGF	2·12±0·17§§§ (16)	258±19§§§ (16)	285±94 (4)
TGF-β1	0·48±0·08§§§ (9)	133±23 (9)	243±16 (4)
IGF-1	1.66±0.24 (10)	276±45§ (10)	224±12 (4)
TPA	2·53±0·25§§§ (4)	533±104§ (4)	432±48§§ (4)

Experiments were performed as described in the text using 20 follicles in duplicate from each rat. Statistical analysis of data was carried out using Student's *t*-test.

†Indicates values significantly different from serum free medium (†$P<0·05$, ††$P<0·01$, †††$P<0·001$).

§Indicates values are significantly different from 1% FCS (§$P<0·05$, §§$P<0·01$, §§§$P<0·001$).

IGF-1, which is secreted into serum-free medium by dermal papilla cells in biologically significant amounts,[15] had no significant effect on the rates of hair follicle elongation, [methyl-^{3}H] thymidine uptake or hair follicle ATP content, but significantly stimulated [U-^{14}C] leucine uptake, indicating that IGF-1 may also be important in hair follicle keratinocyte maturation. We have previously reported similar observations for maintained human hair follicles.[7]

When freshly isolated rat hair follicles are maintained *in vitro* for 24 h in the presence of EGF, there is a significant increase in the rate of [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake, but EGF significantly inhibited the rate of hair follicle elongation after 24 h in culture. These observations, although conflicting, are consistent with *in vivo* observations. EGF stimulates the thickening of neonatal epidermis *in vivo*,[16] and inhibits hair growth in mice and sheep by inducing premature entry into a catagen-like state.[17,18] It has been shown[19] that at high concentrations *in vitro*, EGF inhibits DNA synthesis in chick embryo epidermal explants, and more recently[20] that different components of the epidermis exhibit different responses to infusions of EGF. The basal cells of the epidermis and the sebaceous glands show an increase in their mitotic index, indicating that EGF is acting as a mitogen on these cells, whereas the basal cells of the hair follicle show a reduction in their mitotic index, indicating that for these cells EGF is inhibitory. It is therefore most likely that in our system the matrix cells of the hair follicle are inhibited by EGF, resulting in the inhibition of hair follicle elongation, whereas the cells of the ORS of the hair follicle are stimulated by EGF, which in itself would not have an effect on hair follicle elongation, but would explain the increase in thymidine and leucine uptake. In isolated human hair follicles[7] the most striking effect of EGF was to switch the hair follicles prematurely into an apparent catagen-like state; this was not apparent in the isolated rat hair follicles, although because of the small size of the hair follicles detailed histology may be required.

When topically applied to the skin of shaved mice, the tumour-promoting phorbol ester TPA has been shown to stimulate hair regrowth by the premature switching of follicles from the telogen to anagen phase of their growth cycle.[21,22] *In vitro*, however, TPA significantly inhibited anagen hair follicle elongation, but stimulated [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake as well as hair follicle ATP content. This observed stimulation of these parameters may reflect TPA stimulation of outer root sheath cell division. There would also appear to be a species difference in the effect of TPA on hair follicles, in that TPA has stimulatory effects on [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake and hair follicle ATP content in isolated rat hair follicles, but inhibits these parameters in isolated human hair follicles.[7] It has recently been reported that TPA stimulates murine keratinocytes but inhibits human keratinocyte cell proliferation.[23]

In summary, we have shown that rat hair follicles can be isolated by micro-manipulation and can be maintained *in vitro* for up to 72 h, during which time they continue to produce a keratinized hair shaft. *In vitro* these hair follicles respond to a number of growth regulatory factors, and this model may prove useful in studying aspects of hair follicle biology. The failure of the hair follicles to grow beyond 72 h would indicate that either the culture medium is lacking in some essential supplement or that, because of the small size of rat hair follicles, the trauma of isolation results in the eventual inhibition of follicle growth. This requires further investigation.

Acknowledgment

We thank Unilever Research for financial assistance.

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British Journal of Dermatology (1992) 127, 608–613.

Recurrent annular erythema associated with anti-SS-B/La antibodies: analysis of the disease-specific epitope

Y.HOSHINO, T.HASHIMOTO, T.MIMORI,* M.AKIZUKI* AND T.NISHIKAWA

*Departments of Dermatology and *Internal Medicine, Keio University School of Medicine, Tokyo, Japan*

Accepted for publication 9 March 1992

Summary We have found that anti-SS-B/La antibodies are present in a group of patients with a characteristic recurrent annular erythema, and immunological abnormalities. The presence of a disease-specific epitope for this entity has been examined by comparing immunological reactivity of anti-SS-B/La antibodies between these patients and patients with classical Sjögren's syndrome who have anti-SS-B/La antibodies but do not have this characteristic erythema.

In immunoblotting using three different antigen sources, all the sera from both groups exhibited the same reaction patterns. V8 protease mapping showed that the sera could be divided into three groups by the difference in reactivity to two main digested peptide fragments. However, no significant relationship of the reactivity in peptide mapping between the two patient groups was observed.

Further studies are necessary to elucidate the role of anti-SS-B/La antibodies in disease pathogenesis in these patients, in particular by more precise peptide mapping using recombinant SS-B/La antigen proteins.

Anti-SS-A (Ro) and anti-SS-B (La) antibodies are detectable in the sera of patients with various collagen diseases, and are known to be specific to Sjögren's syndrome.[1–5] Following the report by Rowell *et al.*[6] of lupus erythematosus with erythema multiforme-like skin lesions, a number of cases of annular erythema associated with various immunological abnormalities, in conjunction with anti-SS-A and anti-SS-B antibodies, have been described.[6–14] Although the relationship between subacute cutaneous lupus erythematosus and anti-SS-A antibodies has been well documented,[7,8,10] a relationship between annular erythema and anti-SS-B antibodies has also been reported.[6,12,15] All four cases reported by Rowell *et al.* had anti-SS-B antibodies.[6] We have recently found that anti-SS-B antibodies are frequently detected in the sera of patients with a characteristic recurrent annular erythema, who exhibit various immunological abnormalities more consistent with Sjögren's syndrome than lupus erythematosus.[15] In fact, recent reports from Japan[12,13] and Germany[14] suggest an association of annular erythema with Sjögren's syndrome.

A number of immunological and biochemical studies have shown that several different epitopes are present on the SS-B antigen molecule, indicating that there may be some epitopes specific to different diseases.[16–22]

In the present study, in order to discover the disease-specific epitope in these patients with annular erythema, we have characterized their sera by immunological methods, including protease peptide mapping.

Methods

Sera

Sera were obtained from 19 patients who showed characteristic annular erythema as well as immunological abnormalities. These patients (five males, 14 females; age range 22–56 years, mean 37·9 years) had been clinically diagnosed as suffering from erythema associated with Sjögren's syndrome, or SCLE.[7,8] The diagnosis of Sjögren's syndrome was confirmed using previously published criteria, including histology of salivary gland tissue and a combination of clinical data with objective evidence of glandular dysfunction.[23] Cutaneous manifestations in these patients were of three types: I, scaly annular erythema with central pigmentation or depigmentation—corresponding to the annular polycyclic type of SCLE (Fig. 1a); II, oedematous, exudative and urticaria-like erythema without desquamation or pigmentation—designated erythema asso-

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Correspondence: Dr T.Nishikawa, Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160, Japan.

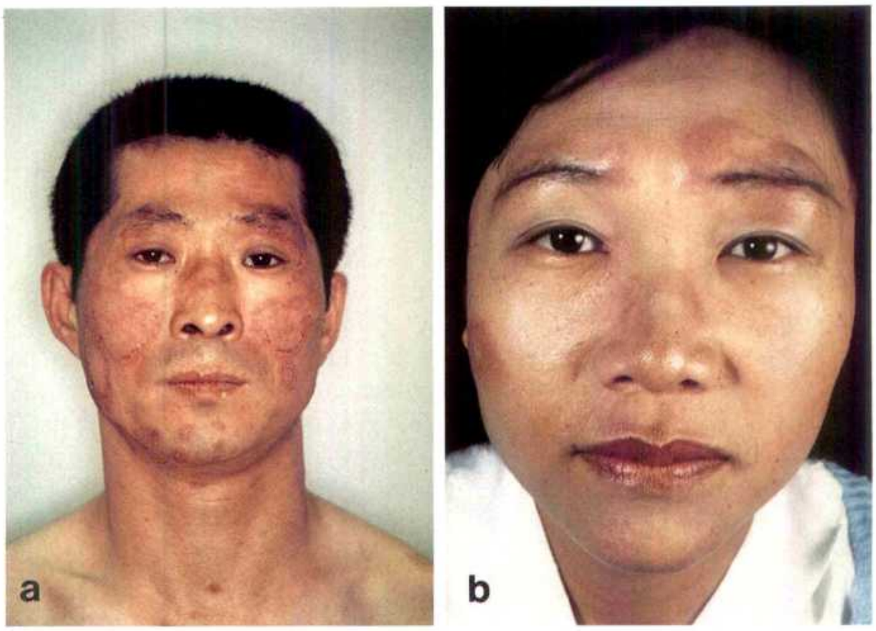

Figure 1. (a) Typical rash of type I, corresponding to the annular polycyclic erythema seen in SCLE, and (b) typical rash of type II, corresponding to erythema associated with Sjögren's syndrome.

ciated with Sjögren's syndrome (Fig. 1b); III, features intermediate between types I and II.

Sixteen patients with positive anti-SS-B antibodies who showed typical sicca symptoms, but had no history of erythema, were selected as controls (two male, 14 female; aged 27–58 years, mean age 47.6). Sera were also examined from four patients who were positive for anti-SS-A antibodies only, and from 10 normal volunteers. The sera were stored at −20°C until used.

Antigens

Rabbit thymus extracts were prepared by the standard method.[24] Human spleen extracts were obtained from Special Reference Laboratories Co. Ltd, Tokyo, Japan.

Confluent cultures of HeLa cells were harvested by scraping with a rubber policeman, and extracted with Tris-HCl buffer, pH 7·2, containing 0·5% NP-40, by sonication. After centrifugation at 10,000 *g* for 10 min, the supernatant was used as crude HeLa cell extract. SS-B antigen was further purified from crude HeLa cell extract.[15] Two affinity columns were prepared using CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and IgG fractions purified by DE52 Cellulose column (Whatman BioSystems Ltd, U.K.) from a serum with a high titre of anti-SS-A antibodies and a serum with high titres of anti-SS-A and anti-SS-B antibodies, according to the manufacturer's recommendations. Crude HeLa cell extract was first added to the column coupled with anti-SS-A antibodies, and the uncoupled fraction was then added to the column coupled with anti-SS-A and anti-SS-B antibodies. SS-B antigen bound to the column was eluted according to the manufacturer's instructions, precipitated with trichloroacetic acid, extracted with acetone, and stored at −20°C.

Immunodiffusion

An immunodiffusion assay was performed by the standard method, using as antigen sources porcine spleen extract for anti-SS-A antibodies and rabbit thymus extract for anti-SS-B antibodies.[3,4,24] Two well-characterized sera, which were specific either for SS-A or SS-B antigen, were used as standards.

V8 protease peptide mapping

The peptide mapping assay was performed by the method of Chan *et al.*[16] Briefly, the affinity-purified SS-B antigen fraction was digested with 0·003% *S. aureus* V8 protease (Boehringer, Mannheim, Germany) in 200 mM Tris-HCl, pH 6·9, containing 2 mM EDTA at 37°C for 30 min. After adding the same volume of twofold concentrated Laemmli's sample buffer[25] (0·0625 M Tris-HCl, pH 6·8, 2% SDS) and boiling for 2 min, the sample was separated by SDS-PAGE and analysed by immunoblotting (described below).

Immunoblotting

SDS-PAGE was performed by Laemmli's method.[25] As antigen sources, we used crude V8 protease-digested SS-B antigen, HeLa cell extract, rabbit thymus extract and human spleen extract to detect anti-SS-B antibodies, after boiling with Laemmli's sample buffer for 2 min. After the separated proteins were transferred to nitrocellulose sheets,[26] blots were first blocked with 3% skimmed milk in TBS (Tris-HCl-buffered saline). Blots were incubated with sera diluted at 1:20, and subsequently incubated with peroxidase-conjugated anti-human IgG antisera diluted 1:100. After each incubation, blots were rinsed for 15 min with three changes of TBS containing 0·5% Tween 20. Finally, antibody-reacted protein bands were detected by colour development with 4-chloro-1-naphthol in the presence of hydrogen peroxide.

Statistical analysis

Statistical analysis was performed by Fisher's test.

Results

Clinical manifestations

Clinical features and laboratory data in patients with and without erythema are summarized in Table 1. In general, both groups of patients showed similar clinical manifestations. The frequency of objective signs of sicca syndrome, arthralgia, leucopenia, hypergammaglobulinaemia and the presence of rheumatoid factor were not significantly different between the two groups. However, subjective sicca symptoms were seen predominantly in the patients without erythema. Moreover, Raynaud's phenomenon was noted in only one patient with erythema, whereas it was present in five patients without erythema.

Table 1. Clinical manifestations and abnormal laboratory data in patients with and without erythema

	Patients with erythema ($n=19$)	Patients without erythema ($n=16$)
Sicca symptoms		
Subjective	6	13
On examination	14	16
Arthralgia	8	11
Raynaud's phenomenon	1	5
Leucopenia	11	9
Hypergammaglobulinaemia	16	15
RA factor	16	14

Anti-SS-A antibodies

By immunodiffusion using porcine spleen extract, anti-SS-A antibodies were detected in 18 of 19 patients with erythema, and in all 16 patients without erythema.

Anti-SS-B antibodies

By immunodiffusion using rabbit thymus extract, anti-SS-B antibodies were detected in all patients at titres ranging from 1:1 to 1:1024.

Because reactivity of anti-SS-B antibodies is known to differ between species[1-4] we performed immunoblotting using different antigen sources, namely, HeLa cell extract, rabbit thymus extract and human spleen extract. Standard serum for anti-SS-B antibodies labelled a 48-kDa protein band in HeLa cell extract, triplet protein bands of 46, 40 and 39 kDa in rabbit thymus extract, and triplet protein bands of 42, 41 and 40 kDa in human spleen extract (Fig. 2). All the sera from the two patient groups (with or without erythema) showed exactly the same reaction patterns in this standard immunoblotting (data not shown).

V8 protease peptide mapping

Immunoblot assay using affinity-purified HeLa cell

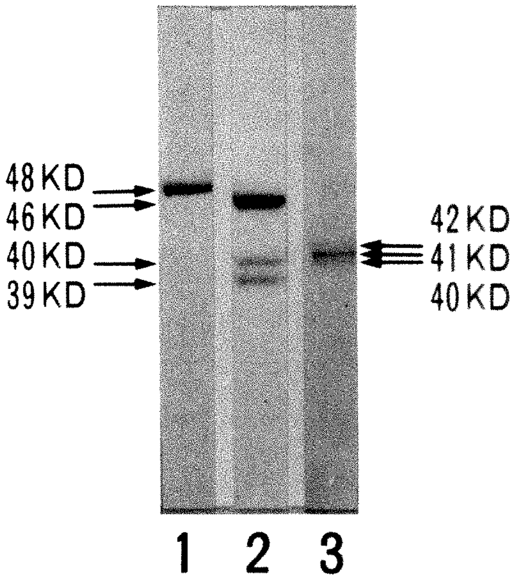

Figure 2. Results of immunoblotting of standard serum for anti-SS-B antibody, using three different antigen sources: lane 1, HeLa cell extract; lane 2, rabbit thymus extract; lane 3, human spleen extract.

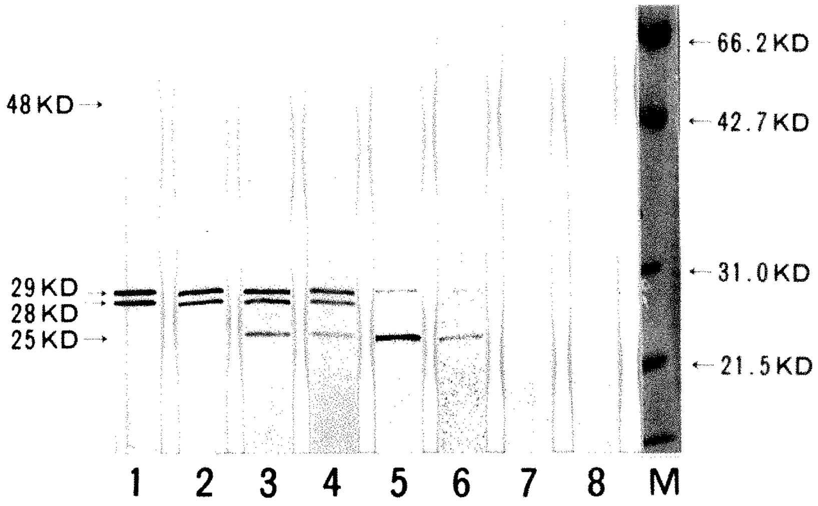

Figure 3. Results of V8 protease peptide mapping. Lanes 1–6: anti-SS-B positive sera. Representative blots of group A (lanes 1 and 2), group B (lanes 3 and 4), and group C (lanes 5 and 6). Lane 7, serum positive for only anti-SS-A antibody; lane 8, control serum. Lane M indicates migration of molecular weight markers.

extract digested with *S. aureus* V8 protease as an antigen source, revealed that anti-SS-B antibodies reacted with 29-, 28- and 25-kDa polypeptide bands, in which 29- and 28-kDa polypeptide bands were always detected simultaneously. However, the pattern of reactivity among the patients was heterogeneous, and distinguished three groups: group A, sera reacting more strongly with the 29- and 28-kDa bands than the 25-kDa polypeptide band (Fig. 3, lanes 1 and 2); group B, sera showing similar reactions with all three bands (Fig. 3, lanes 3 and 4); and group C, sera reacting more strongly with the 25-kDa band (Fig. 3, lanes 5 and 6). None of these three polypeptide bands was detected by either sera reacting only with SS-A antigen (Fig. 3, lane 7) or normal sera (Fig. 3, lane 8).

Relationship between the reactivity on peptide mapping and clinical manifestations

Table 2 shows the relationship between the reactivity of anti-SS-B antibodies with digested polypeptide and the presence of erythema. Almost two-thirds of individuals were classified as group A in patients both with and without erythema, and no significant differences were found in frequencies of group B and C in these two patient groups. When the results of peptide mapping were compared among the three different types of annular erythema, i.e. types I, II and III, no significant difference was seen (Table 2). We also examined the relationship between the reactivity on peptide mapping and other clinical features, such as sicca symptoms, arthralgia, Raynaud's phenomenon, leucopenia and hypergammaglobulinaemia, but no significant difference was found (data not shown).

Discussion

We have examined the fine specificities of anti-SS-B antibodies and clinical features of 19 cases with a characteristic recurrent annular erythema and immunological abnormalities. The results of these studies were compared with those in 16 patients who also had anti-SS-B antibodies but did not have erythema.

It has been reported that, although almost all patients with anti-SS-B antibodies also have anti-SS-A antibodies, anti-SS-B antibodies are detected in approximately 20–30% of anti-SS-A-positive patients.[5,12,13] In our present study, all the patients had both anti-SS-B/La and SS-A/Ro antibodies, except one patient whose serum was positive for anti-SS-B/La but not for SS-A/Ro antibodies by immunodiffusion. We did not find any patients with erythema who showed anti-SS-A antibodies alone. From these results, we speculate that the erythema seen in our patients may be associated with anti-SS-B antibodies, rather than with anti-SS-A antibodies.

Table 2. The results of protease peptide mapping in patients with and without annular erythema

	Classification by protease peptide mapping			
	Group A	Group B	Group C	Total
Patients with erythema	13	3	3	19
Type I	3	0	0	3
Type III	2	2	1	5
Type II	8	1	2	11
Patients without erythema	10	5	1	16

However, the biochemical characters of SS-B antigens found in different species are known to vary.[1-4] In our immunoblot analyses using HeLa cell extract, rabbit thymus extract and human spleen extract, SS-B antigen was labelled in a variety of patterns. Furthermore, Chan *et al.* reported that V8 protease digestion of HeLa cell-derived SS-B antigen produces two major polypeptides, 28 kDa (designated 'X') and 25 kDa (designated 'Y'), and the reactivity of anti-SS-B antibodies with these polypeptides is heterogeneous.[16] Because many reports have suggested that there may be disease-specific epitopes or pathogenic epitopes on various autoantigens,[18,20,22,27,28] we speculated that the anti-SS-B antibodies associated with our patients with erythema may recognize specific epitope(s) which might be different from those recognized by anti-SS-B antibodies in patients without erythema.

We examined all sera from both groups by immunoblotting using three different antigen sources. However, all sera reacted with undigested SS-B antigen in exactly the same patterns. We then performed V8 protease mapping using affinity-purified SS-B antigen from HeLa cell extract. We found that all anti-SS-B positive sera labelled a doublet of 29- and 28-kDa proteins (designated 'X' and 'X'', respectively) and a 25-kDa protein (designated 'Y') in a variety of patterns. Recent studies have shown that the polypeptides X and X′ correspond to the amino-terminal half of the native SS-B molecule, and polypeptide Y corresponds to the carboxy-terminal half.[16] Our results indicate that at least one epitope is present both in the X/X′ and Y portions of the molecule and that the reactivity of each serum is heterogeneous. We arbitrarily divided the patients into three groups, i.e. A, preferentially reacting with X and X′, B, reacting equally with all three polypeptides and C, preferentially reacting with Y, and the patients were classified according to these criteria. The fact that approximately two-thirds of sera preferentially reacted with X/X′ indicates that major epitope(s) may reside in this area of the SS-B antigen. In order to examine the epitopes specific to the patients with erythema, groups with and without erythema were compared. However, there was no significant difference between these groups.

The results in the present study did not suggest the presence of a disease-specific epitope in the patients characterized by annular erythema and anti-SS-B/La antibodies. This may indicate that other factors are responsible for the expression of erythema in this patient group. However, recent molecular biological techniques have enabled us to use recombinant SS-B proteins which can be divided into more restricted regions of the molecule.[17-20] To elucidate a real association of anti-SS-B antibodies with annular erythema, more precise epitope mapping using these recombinant proteins will be necessary in future studies.

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British Journal of Dermatology (1992) 127, 614–619.

DERMIS: a computer system for assisting primary-care physicians with dermatological diagnosis

G.J.BROOKS,* R.E.ASHTON† AND R.J.PETHYBRIDGE‡

Royal Naval Medical Service,† Royal Naval Hospital Haslar, ‡Institute of Naval Medicine, Gosport, Hampshire PO12 2AA, U.K.

Accepted for publication 26 June 1992

Summary

DERMIS is a computerized skin disease diagnostic prompting system which has been derived from the prospective study of 5203 cases. It has been designed for use by non-dermatologists such as general practitioners. The program produces a list of reasonable diagnoses based on probabilities calculated using Bayes' theorem. Out of 221 precise diagnoses made by the dermatologist in the clinic, 42 groupings were created to encompass the most common or important diseases encountered in general practice. Four 'remainder' or 'send to specialist' groups were included for the 13% of uncommon conditions. The program, when tested by 'one out' analysis on the original cases, placed the correct diagnosis first on 76% of occasions, and within the first three on 95% of occasions. In 76 of 125 cases randomly selected from the data base, a request for diagnostic assistance had been made by the referring general practitioner. It has been estimated that in 54 of these 76 cases the DERMIS system could have provided differential advice with the correct diagnosis appearing at the top of the list.

The system has been written in *MUMPS* and runs on an IBM-compatible desk-top computer. The software allows real time data entry. Arrangements are in hand for performing clinical trials of the system in general practice. If current performance is maintained, and the response to the system's output is favourable, then DERMIS might well enhance performance in medical decision making for the benefit of patients, medical services and budget holders.

During the last two decades, numerous computer programs have been designed to offer medical advice on a variety of topics.[1,2] Unfortunately, the creator's enthusiasm has rarely been matched by any compelling medical need for the product. Even when projects have appeared appropriate, many have failed to find widespread use, as there has been no easy way of implementing them. The current emphasis on audit and budget control has led to the installation of computer terminals in many general practitioners' consulting rooms. The hardware is being used to store clinical information and could also run diagnostic prompting software.

Skin disease accounts for about 7% of all consultations in general practice, yet many doctors find the accurate diagnosis of skin disease difficult. This should not be the case, as skin physical signs are visible for all to see. Standard terminology can be used to describe the physical signs, but the interpretation of these is learned by experience and not from standard textbooks. The objective of this study has been to produce a tool which can analyse physical signs and assist with the formulation of a differential diagnosis.

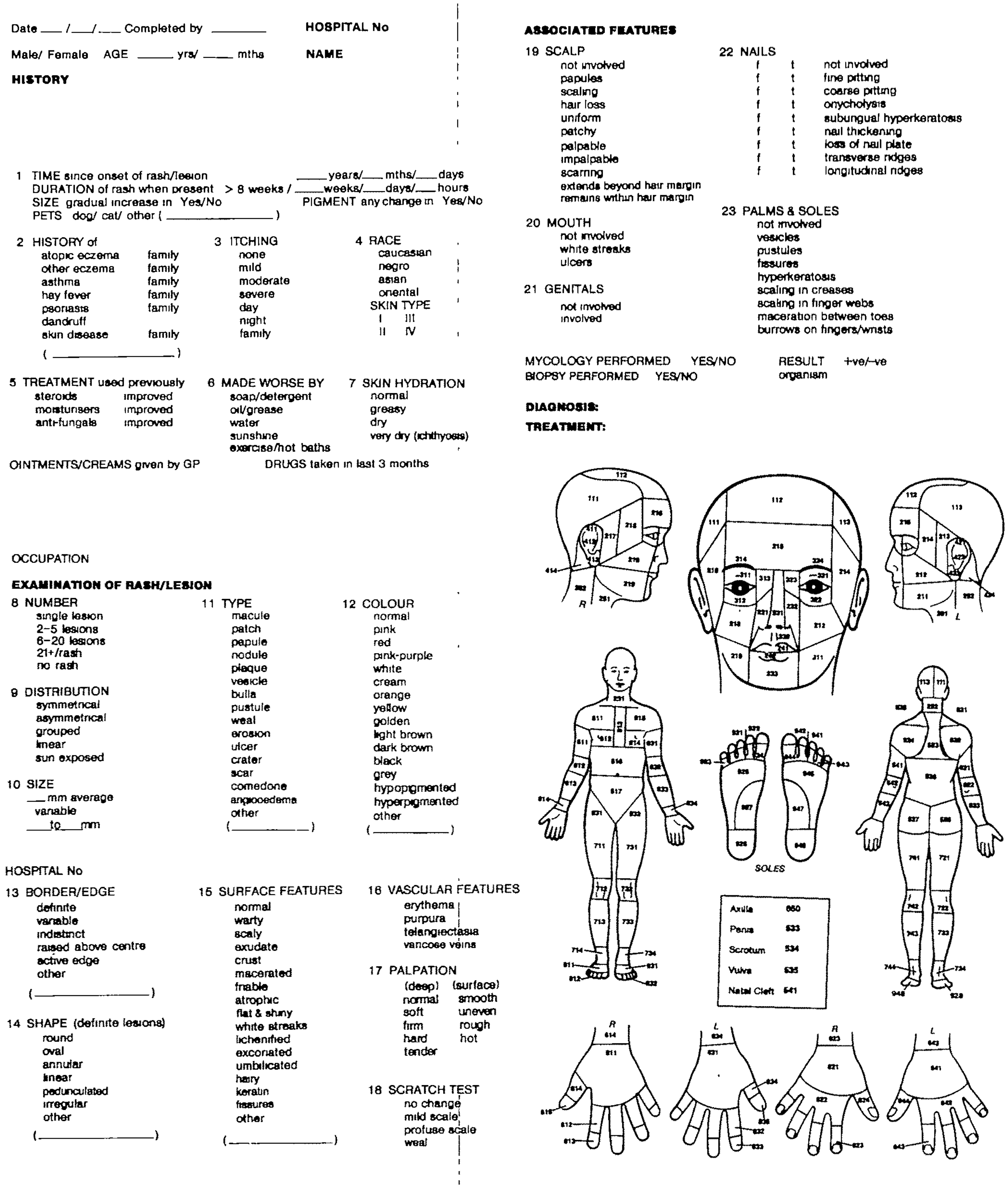
The potential value of the prompting system to users has been assessed from a sample of cases by analysing the reason for referral, to identify whether diagnostic advice or treatment is requested.

Methods

The subjects included 5203 consecutive patients (new referrals only) seen by a dermatologist (R.E.A.) from 1985 to 1989. Any omissions occurred randomly due to logistic reasons. Data was collected from each case according to a comprehensive list of dermatology symptoms and signs (Fig. 1). Data collection was performed by the consultant, or clinical assistant/general practice trainee. Signs and symptoms were, however, checked by the consultant for consistency. The terms employed had previously been defined and tested through practical use by students and general practitioner trainees. The collection sheet was self-copying and was in two parts. One of these became the patient record. The other was coded to allow data base entry by staff of the Institute of

Correspondence: Surgeon Commander R.E.Ashton RN. Dermatology Department, Royal Naval Hospital, Gosport, Hampshire PO12 2AA, U.K

* Present address. Egton Medical Information Services, 77 Back Lane, Horsforth, Leeds LS18 4RF, U.K.


R
L
SOLES
R
L
R
L

Date ___ /___/___ Completed by ________ **HOSPITAL No**

Male/ Female AGE _____ yrs/ ____ mths **NAME**

HISTORY

1 TIME since onset of rash/lesion _____years/____mths/____days
DURATION of rash when present > 8 weeks / _____weeks/____days/____hours
SIZE gradual increase in Yes/No PIGMENT any change in Yes/No
PETS dog/ cat/ other (______________)

2 HISTORY of

atopic eczema	family
other eczema	family
asthma	family
hay fever	family
psoriasis	family
dandruff	
skin disease	family

(______________)

3 ITCHING
none
mild
moderate
severe
day
night
family

4 RACE
caucasian
negro
asian
oriental
SKIN TYPE
I III
II IV

5 TREATMENT used previously

steroids	improved
moisturisers	improved
anti-fungals	improved

6 MADE WORSE BY
soap/detergent
oil/grease
water
sunshine
exercise/hot baths

7 SKIN HYDRATION
normal
greasy
dry
very dry (ichthyosis)

OINTMENTS/CREAMS given by GP DRUGS taken in last 3 months

OCCUPATION

EXAMINATION OF RASH/LESION

8 NUMBER
single lesion
2–5 lesions
6–20 lesions
21+/rash
no rash

9 DISTRIBUTION
symmetrical
asymmetrical
grouped
linear
sun exposed

10 SIZE
___mm average
variable
____to___mm

11 TYPE
macule
patch
papule
nodule
plaque
vesicle
bulla
pustule
weal
erosion
ulcer
crater
scar
comedone
angiooedema
other
(__________)

12 COLOUR
normal
pink
red
pink-purple
white
cream
orange
yellow
golden
light brown
dark brown
black
grey
hypopigmented
hyperpigmented
other
(__________)

HOSPITAL No

13 BORDER/EDGE
definite
variable
indistinct
raised above centre
active edge
other
(______________)

14 SHAPE (definite lesions)
round
oval
annular
linear
pedunculated
irregular
other
(______________)

15 SURFACE FEATURES
normal
warty
scaly
exudate
crust
macerated
friable
atrophic
flat & shiny
white streaks
lichenified
excoriated
umbilicated
hairy
keratin
fissures
other
(______________)

16 VASCULAR FEATURES
erythema
purpura
telangiectasia
varicose veins

17 PALPATION

(deep)	(surface)
normal	smooth
soft	uneven
firm	rough
hard	hot
tender	

18 SCRATCH TEST
no change
mild scale
profuse scale
weal

ASSOCIATED FEATURES

19 SCALP
not involved
papules
scaling
hair loss
uniform
patchy
palpable
impalpable
scarring
extends beyond hair margin
remains within hair margin

20 MOUTH
not involved
white streaks
ulcers

21 GENITALS
not involved
involved

22 NAILS

f	t	not involved
f	t	fine pitting
f	t	coarse pitting
f	t	onycholysis
f	t	subungual hyperkeratosis
f	t	nail thickening
f	t	loss of nail plate
f	t	transverse ridges
f	t	longitudinal ridges

23 PALMS & SOLES
not involved
vesicles
pustules
fissures
hyperkeratosis
scaling in creases
scaling in finger webs
maceration between toes
burrows on fingers/wrists

MYCOLOGY PERFORMED YES/NO RESULT +ve/–ve
BIOPSY PERFORMED YES/NO organism

DIAGNOSIS:

TREATMENT:

Axilla	660
Penis	533
Scrotum	534
Vulva	535
Natal Cleft	541

Figure 1. Data collection sheet used during patient interview.

Naval Medicine on to a mainframe computer. This information was later transferred to an IBM-compatible desk-top computer for analysis.

The diagnosis in each case was made clinically by the dermatologist at the time of the examination, or modified later based on histological or mycological findings.

Bayes' formula was used to calculate the likelihood of each disease being present given a set of symptoms and signs. The probability of a disease being present (i.e. the computer's suggestion or diagnosis) can be calculated if we know the probability of the disease occurring in the population (here our data base), the probability of the sign occurring in that disease (measured by collecting the cases) and the probability of the sign occurring in all diseases. In mathematical terms this is expressed as the following formula:
If disease D (e.g. psoriasis) and sign S (e.g. scaling) are independent events then:[3]

$$P\,(D\backslash S)=\frac{P\,(S\backslash D)\times P\,(D)}{P\,(S)}$$

where $P\,(D\backslash S)$ is the probability of a disease (e.g. psoriasis) being present given the sign (e.g. scaling) elicited;
$P\,(S\backslash D)$ is the probability of the sign (scaling) occurring in psoriasis;
$P\,(D)$ is the prior probability of the disease occurring, i.e. how commonly psoriasis occurs in the data base;
$P\,(S)$ is the probability of the sign occurring at all.

By using this calculation for each disease in turn, relative likelihoods (as a percentage) of all diseases are produced. The diseases can be placed in order by percentage likelihood to give a differential list.

It was apparent from examining cases that combinations of signs occurred within categories (e.g. two colours or two types of lesion are present). Single probability estimates have been formed for these combinations and used in the Bayes' equation instead of the separate 'independent' component estimates.

There were 221 separate diagnoses. Where appropriate, diagnoses were collated into groups (Table 1). For example, psoriasis has been used as a general term to describe cases of plaque, guttate, intertriginous, nail and scalp psoriasis. For some other diseases it was appropriate to retain the original diagnosis. For example, malignant melanoma is represented in both nodular and superficial spreading forms, as the clinical features are so different. Rare diseases have been represented in four 'remainder' or 'refer to specialist' groups.

Table 1. Accuracy of assignment of correct diagnosis to top of differential list in 'one-out' analysis of 5203 cases in data base

Group title	No. cases data base	No. (%) of times top differential
1. Alopecia areata*	40	39 (98)
2. Acne†	253	232 (92)
3. Basal cell carcinoma	288	201 (70)[a]
4. Superficial BCC	24	23 (96)
5. Bowen's disease	27	22 (81)[b]
6. Chondrodermatitis	31	28 (90)
7. Cyst epidermoid	59	51 (86)
8. Dermatofibroma	90	77 (86)
9. Eczema‡	1124	847 (75)
10. Folliculitis	23	19 (83)
11. Granuloma annulare	47	42 (90)
12. Herpes simplex	23	22 (96)
13. Insect bite§	31	24 (77)
14. Keratoacanthoma	25	22 (88)
15. Lentigo	38	28 (74)
16. Lichen planus¶	64	54 (84)
17. Malignant melanoma	21	18 (86)[c]
18. Malig. melan. superficial	51	49 (96)[d]
19. Milia	23	20 (87)
20. Molluscum contagiosum	47	45 (96)
21. Naevus**	396	250 (63)
22. Naevus spider	36	36 (100)
23. Pyogenic granuloma	47	46 (98)
24. Pityriasis rosea	19	18 (95)
25. Psoriasis palm/plantar	10	10 (100)
26 Psoriasis††	414	379 (91)
27. Pityriasis versicolor	68	54 (79)
28. Rosacea	60	53 (88)
29. Scabies	52	51 (98)
30. Seborrhoeic wart	266	183 (69)
31. Solar keratosis‡‡	269	187 (69)
32 Skin tags	33	32 (97)
33. Squamous cell carcinoma	35	26 (74)[b]
34. Tinea§§	82	64 (78)
35 Urticaria	69	65 (94)
36. Vitiligo	24	23 (96)
37. Warts¶¶	277	222 (80)
38. Verruca***	50	49 (98)
39. Other single lesion	217	109 (50)
40. Other multiple lesions	190	101 (53)
41. Other rash	253	104 (41)
42. Other no rash or lesion	7	6 (86)

* Includes cases of alopecia totalis.
† Includes cases of acne excoriée, perioral dermatitis.
‡ Includes cases of eczema, atopic, contact allergic, discoid, foot and hand, intertriginous, impetiginized, pompholyx, craquelé, seborrhoeic, varicose, papular, acute, irritant; lichen simplex chronicus, juvenile plantar dermatosis.
§ Includes cases of papular urticaria
¶ Includes cases of lichen planus hypertrophic.
** Includes cases of naevus: junctional, blue, compound, halo, hairy, pigmented, intradermal, linear epidermal, warty epidermal.
†† Includes cases of plaque, guttate, intertriginous, nail, scalp
‡‡ Includes cases of cutaneous horn.
§§ Includes cases of tinea: corporis, cruris, incognito, manuum, pedis, unguium.
¶¶ Includes cases of common, filiform, genital, plane.
*** Includes cases of corn, exostosis.
[a] Not included 49 cases diagnosed as other malignancy.
[b] Not included four cases diagnosed as other malignancy.
[c] Excluding one case diagnosed as superficial spreading melanoma.
[d] Not included one case diagnosed as other malignancy.

A 'one-out' method was adopted[4] to test the accuracy of the computer model. Each case in turn was excluded from the data base, and the frequency of symptoms and signs being present for each diagnosis was calculated using the remaining cases. An estimate of the likelihood of occurrence of each disease with the symptoms and signs of the excluded case was calculated by multiplication of the appropriate frequency and prior probability information. The diseases were then ranked according to their likelihood scores to give a differential list.

Assessment of predictive accuracy using the algorithm involved:

1. the original 221 diagnoses;
2. 42 diagnostic groups created using the diagnosis reached in 1;
3. combination of diagnoses into 42 groups before calculation using the algorithm;
4. as for 3, using a reduced data sheet.

Where the predicted diagnosis has disagreed with the actual diagnosis, the computer's differential diagnosis was investigated in order to assess where the correct diagnosis appeared in the differential list.

The clinical records from a random sample of 125 collected cases were scrutinized to determine the reason for referral, in order to determine when a diagnostic advice system might be of use.

Results

Within the data base the number of cases allocated to each of 221 specific diagnoses varied considerably, from the hundreds of 'eczema' cases to one or two representatives of extremely rare diseases. The diagnosis appearing at the top of the differential was correct in 60% of cases when all 221 diagnoses were used. However, if a correct diagnosis was more loosely confined to one of the 42 major diagnostic groups, the overall predictive value increased to 76%. In a further test, where the 42 groups were formed prior to use of the predictive algorithm, the diagnostic accuracy was again found to be 76%, but the pattern of success and failure of individual cases varied between the two methods. On 95% of occasions the correct diagnosis appeared in the top three of the differential list.

The original data sheet (Fig. 1) allowed 270 options for description of cases. The list was reviewed in the light of clinical experience and analysis of the predictive value of each item. It was found that the list could be reduced to 115 items without loss of predictive accuracy.

Table 2. Most common errors: confusion between specific disease groups

Actual diagnosis	Top of differential	Number of cases (%)
Eczema	Psoriasis	84 (7)
Send to specialist	Eczema	70 (3)
Eczema	Scabies	63 (6)
Eczema	Tinea	38 (3)
Naevus	Seborrhoeic wart	21 (5)
Eczema	Pityriasis rosea	20 (2)
Eczema	Send to specialist	20 (2)
Naevus	Superficial melanoma	18 (5)
Basal cell carcinoma	Squamous cell carcinoma	18 (6)
Naevus	Skin tag	18 (5)
Send to specialist	Pityriasis versicolor	17 (7)
Seborrhoeic wart	Naevus	15 (6)
Acne	Rosacea	15 (6)

The accuracy with which the diagnosis was predicted varied between the groups (Table 1). For example, 847 (75%) of the 1124 cases of eczema were correctly identified by the program, compared with 49 (96%) of the 51 cases of superficial spreading melanoma. Cases of rarer diseases, e.g. pemphigoid, mycosis fungoides, which were assigned to 'remainder' groups, made up 13% of the total database. These groups had the highest failure rates, i.e. 41–53% (Table 1).

The system output was studied in order to determine any patterns of success and failure. Certain diseases tended to be confused with one another (see Table 2). For example, the commonest type of failure occurred in 84 cases of eczema where the system placed psoriasis above eczema in the differential list. In 49 cases of the papular/nodular presentation of basal cell carcinoma, a malignant tumour other than basal cell carcinoma (or 'remainder' group in four cases) was selected by the system. The management of these cases is the same—either excision biopsy or referral to a specialist.

A sample of 125 randomly selected case records were scrutinized in detail, in order to determine the reason for referral and the outcome following the clinic visit. The results are presented in Table 3. In 76 (61%) cases (summarized in Table 4) the family practitioner requested assistance with diagnosis. The final diagnosis by the dermatologist matched one of the 38 main DERMIS disease group headings in 71 (93%) of these cases. The number of cases in which the DERMIS system placed the correct diagnosis at the top of its differential list was 54 (71%).

Table 3. A random sample of 125 cases referred to dermatology clinics by primary care physicians: reason for referral vs outcome

	Outcome			
Reason for referral	Malignant tumour	Benign lesion(s)	Rash no infection	Infection/ infestation
Diagnosis and management				
(a) diagnosis unknown	5*	19*	21*	7*
(b) ? malignant	6*	11*	3*	—
(c) ? infection	—	—	3*	—
Diagnosis correct, for second opinion	—	4*	2*	1*
Management only	—	4	2	3
Re-referral, further treatment	—	—	15	9
Removal/biopsy	—	10	—	—

* Indicates the cases shown in Table 4.

Table 4. Breakdown by diagnosis of 76 randomly selected cases referred by primary care physicians to dermatology clinics for diagnosis and management

Final diagnosis	Number of cases
Basal cell carcinoma	9
Bowen's disease	1
Discoid lupus	1
Eczema	15
Erysipelas	1
Insect bites	2
Lentigo	2
Lichen planus	2
Naevus	12
Rare tumours	2
Pyogenic granuloma	1
Pityriasis rosea	1
Psoriasis	4
Pityriasis versicolor	1
Rosacea	1
Scabies	2
Solar keratosis	7
Squamous cell carcinoma	1
Tinea	5
Urticaria	2
Vasculitis	1
Viral wart	1
Verruca	2

Discussion

Prospective collection of case data is one of the techniques commonly used to acquire knowledge for computer-based medical prompting and expert systems. The method relies on the clinician defining the diagnosis and ensuring that all relevant information is collected. Case collection is a lengthy task which needs to be carefully planned and conscientiously carried out before any predictions can be made. However, the effort frequently brings rewards as previous gaps in knowledge are filled and uncertain boundaries between diseases are clarified.

Haberman *et al.*[5] chose not to use case collection in creating a diagnostic system for dermatologists. Instead, they asked dermatologists to estimate the importance of various clinical features in diseases. These estimates were then used as the basis of weightings and rules which formed the diagnostic algorithm. The system which they produced has been tested, with mixed results. In the later stages of development case information was fed back into the system in order to adjust the original weightings. Other methods which have been used in attempts to create diagnostic advice systems for dermatology have been described by Stoecker.[6]

The statistical approach to pattern matching adopted in this study has been popular for many years. The requirements, advantages and disadvantages of Bayesian methodology are well known.[7] There are several sources of variation which are likely to determine the performance of the algorithm in practical use. Disease prevalence can alter with geographic area. If DERMIS was to be tested in general practice, appropriate prevalence rates would have to be used to prime the system.

The presentation of a disease can vary between patients, and the content of data collected from the same patient can vary between doctors. In this study the cases were all collected by one of the authors (R.E.A.), and occasionally by GP trainees or medical students. There was no allowance for inter-observer variation.

A further weakness in this study has been that the dermatologist (R.E.A.) could have introduced substantial bias by subconsciously fitting symptoms and signs to diseases rather than faithfully recording new case details. This could lead to increased accuracy of the program when tested against itself. Testing the program against other dermatologists would help reveal any bias.

Data has been collected mainly from white Caucasian patients. Rashes in patients with pigmented skin may look different, but although the colour of the rash may be different, other features such as surface changes and border characteristics should be the same.

The accuracy of DERMIS using the original 221 diagnoses is comparable with similar systems in other subjects.[7] Many of the errors were eliminated by combining different forms of the same disease (i.e. combining all the eczemas), to produce a predicted accuracy of 95% for the first three in the differential list. It has not been possible to eliminate all DERMIS system errors by the combination of subgroups. The clinical appearance of eczema, for example, can be identical to that of tinea infection, and that of a solar keratosis visually similar to a basal cell carcinoma. Combining two such diseases into the same group is of no immediate value to any system user, as the treatments are different. In these cases a dermatologist would investigate further to establish the diagnosis. The prompting system could be set to recommend further investigation when two diseases are known to cause problems.

The group combinations of Table 1 were tested in two ways. Combining these before application of the algorithm led to a dilution effect of the important diagnostic features of the subgroups. Whereas, combination of the groups following application of the algorithm led to under-diagnosis of the small subgroups. The outcome of the two types of combination was virtually the same, with an overall diagnostic accuracy of 76%.

The accuracy with which the correct diagnosis was placed at the top of the differential list by the system appeared to be related both to group size, and variability of presentation. Increasing the number of cases of a disease in the data base will improve the accuracy with which the disease is identified. However, if there are few distinctive features, or the presentation varies from case to case, then performance will be degraded. In such cases dermatologists often have a distinct advantage, as their expertise enables them to sift the available information for important clues which may have variable weighting according to circumstance.

DERMIS was not designed to replace dermatologists, but to offer general practitioners assistance with the formulation of a reasonable differential diagnosis. The system was able to place the correct diagnosis in the top three of its differential output on 95% of occasions. It also demonstrated a high accuracy in identification of malignancy, even if the correct tumour was not always placed at the top of the list. It is important to recognize that computer-based diagnostic prompting or expert systems are unlikely ever to recommend the correct diagnosis on all occasions.

When a random sample of 125 cases from the data base was reviewed it was found that 76 (61%) had been referred for initial diagnosis or a second opinion. The system offered a differential listing with the correct diagnosis at the top in 54 of these 76 cases. It is not known what influence this type of information would have on referral patterns.

Currently, patients may have to wait many months before being seen in a dermatology clinic. Use of a system such as DERMIS might encourage general practitioners to treat more cases themselves and to make more appropriate referrals.

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British Journal of Dermatology (1992) 127, 620–624.

The influence of food on 8-methoxypsoralen serum concentration and minimal phototoxic dose

N.BECH-THOMSEN,* H.R.ANGELO† AND E.A.KNUDSEN‡

**Laboratory of Photobiology, Department of Dermatology, Rigshospitalet, University of Copenhagen, Denmark*
Departments of †Clinical Chemistry and ‡Dermatology, Bispebjerg Hospital, University of Copenhagen, Denmark

Accepted for publication 8 June 1992

Summary

Ten adult volunteers were given three oral doses of 0·46–0·56 mg/kg body weight of 8-methoxypsoralen (8-MOP) in a liquid formulation under fasting conditions, and after ingestion of a low-fat or fat-rich breakfast. 8-MOP serum levels and photosensitivity were measured 0·5–4 h after drug ingestion. The 1-h 8-MOP serum levels and photosensitivity were significantly higher in fasting conditions than after ingestion of a low-fat or fat-rich meal (intra-individual median difference in photosensitivity 7·5 J/cm^2). On 12 of 20 occasions when the drug was taken after food ingestion, the 1-h 8-MOP serum concentration was below 30 ng/ml.

A survey of 43 out-patients undergoing regular PUVA treatment showed that the frequency of erythemal reactions was significantly higher when 8-MOP was ingested with a $>50\%$ smaller quantity of food than usual ($P<0{\cdot}005$).

This study demonstrated food-induced variations in 8-MOP photosensitivity both in an experimental situation and in an out-patient survey. In order to optimize the therapeutic effect of PUVA, the quantity of food taken before 8-MOP should remain constant, and the timing of UVA irradiation should be adjusted according to the preceding food intake.

We use a liquid formulation of 8-MOP (Geroxalen, Gerot, Austria) for PUVA treatment. This produces a rapid peak in the 8-MOP serum concentration, and a short period of photosensitivity.[1] In Denmark it is recommended that UVA radiation is administered 1 h after 8-MOP ingestion, and this recommendation is based on the use of 8-MOP in a liquid formulation. Using this regimen, we have observed that some PUVA out-patients develop marked erythema. The 8-MOP serum concentration was determined on consecutive treatment days in seven patients, and this revealed that the intra-individual variation in 8-MOP serum levels at the time of irradiation reached a factor of 10 in some of the patients.

Two previous studies have investigated the effect of food on the kinetics of a liquid formulation of 8-MOP. In the first of these, the effect of food was not evaluated statistically, but preceding or concomitant food intake did not appear to influence serum drug levels.[1] In the second study, the degree and time of peak photosensitivity was not affected by the fat content of the breakfast ingested before 8-MOP administration.[2]

We report a study in which a liquid formulation of 8-MOP was taken under three different conditions: fasting, after a low-fat breakfast, and after a fat-rich breakfast. The 8-MOP serum level, and photosensitivity, were measured at intervals over a 4-h period after drug ingestion. In addition, we carried out a survey of out-patients undergoing regular PUVA treatments to evaluate the influence of variations in the patients' dietary intake on the occurrence of adverse erythemal reactions during PUVA treatment.

Methods

Experimental study

Subjects. Ten healthy volunteers (age range 23–66 years; skin types II and III) were enrolled in the study after giving informed consent. The study was approved by the Medical Ethics Committee of Copenhagen.

Protocol. Each volunteer took 8-MOP (0·46–0·56 mg/kg body weight) on three separate mornings after fasting overnight. 8-MOP was taken while fasting, 1 h after a low-fat breakfast, and 1 h after a fat-rich breakfast. The order of the different regimens was randomly chosen.

Correspondence. Dr N Bech-Thomsen, Laboratory of Photobiology, Department of Dermatology, Rigshospitalet H-5192, Blegdamsvej 9, DK-2100 KBH Ø, Denmark.

More than 3 days elapsed between each test. The fat-rich breakfast consisted of 100 g bread, 90 g cheese, 25 g butter, 0·5 l milk and 0·15 l coffee (73·5 g fat, 74·5 g carbohydrate, 45 g protein). The low-fat breakfast consisted of 100 g bread, 100 g marmalade, 0·25 l orange juice and 0·15 l coffee (3 g fat, 130 g carbohydrate, 11 g protein).

Assay of 8-MOP. Ten-millilitre samples of venous blood were collected before and 0·5, 1, 1·5, 2, 3 and 4 h after each drug ingestion. The serum fraction was separated and stored at −20°C. The concentration of 8-MOP was determined by a modification of the HPLC method described by Ketchum *et al.*[3] One-millilitre of serum was extracted with 5 ml toluene containing the internal standard (Imperatorin [Serva]) for 10 min. After centrifugation, the organic phase was transferred to a new glass tube and evaporated to dryness at 50°C under nitrogen. The residue was dissolved in 200 μl mobile phase, and 80 μl was analysed by HPLC. The analytical column was a 5-μm LiChrospher 100 RP-18 e 25 cm × 4·6 mm ID (Merck) used at room temperature, with a mobile phase of acetonitrile–water (55:45). Using a model F 1000 Hitachi fluorescence detector, operating at 313 nm (excitation) and 480 nm (emission), the lower limit of quantitation was 20 ng/ml. The interassay coefficient of variation was 7·5% (300 ng/ml).

Photosensitivity testing. Photosensitivity was tested 0·5, 1, 1·5, 2, 3 and 4 h after ingestion of 8-MOP, by exposure to a UVASUN 2000 lamp (Mutzhas, Germany). At each time point a dose of 3, 6, 9, 12 and 15 J/cm^2 of radiation in the range 335–380 nm was given to five circular test sites (1 cm diam.). Each volunteer was tested on the lateral aspect of the thigh, the abdomen, and the lower back. The order of the three sites was randomly chosen. The intensity of the UVASUN was measured with a UVX radiometer (Ultraviolet products, U.S.A.) with a UVX-36 detector, and adjusted according to the spectral response curve of the detector and the emission spectrum of the UVASUN. The phototoxic reaction was evaluated clinically at 72 h. The minimal phototoxic dose (MPD) was defined as the minimum dose of UVA required to elicit erythema with well-defined borders.

Out-patient survey

For a period of 18 months information on the type and quantity of beverage and food consumed immediately before and with 8-MOP administration, and the occurrence of erythemal reactions, was recorded after every regular PUVA treatment in our department. The records of 43 patients who received more than 10 consecutive PUVA treatments were examined, in order to determine if the occurrence of adverse erythemal reactions was related to a >50% reduction in the quantity of food consumed before or with 8-MOP.

Statistics

Inter-individual differences in 8-MOP serum levels and MPD with the three dietary regimens were compared using the Mann–Whitney *U*-test. Intra-individual differences were compared using the Wilcoxon test. The differences were considered significant when $P<0{\cdot}05$. To evaluate the photosensitivity during the three 8-MOP regimens, all tests with an MPD above 15 J/cm^2 were given an artificial value from 16 to 18 J/cm^2, according to the reaction at the 15 J/cm^2 test site.

Results

8-MOP serum concentration

The time course of serum drug levels during fasting conditions and after food intake is shown in Figure 1. The 0·5 and 1 h 8-MOP serum levels were significantly higher during fasting than those after a low-fat or fat-rich breakfast. There was no difference in serum levels between the low-fat and fat-rich breakfast. Peak serum concentration (C_{max}) was significantly higher during fasting than after food intake (median intra-individual difference between fasting and low-fat, 280 ng/ml, confidence interval (CI) 75–540 ng/ml, $P=0{\cdot}01$; between fasting and fat-rich breakfast, 400 ng/ml, CI 248–597 ng/ml, $P<0{\cdot}01$). Time to C_{max} was significantly delayed when the drug was taken after a meal compared with the fasting state, $P\leqslant 0{\cdot}01$ (median intra-individual difference 1·5 h). Furthermore, the inter-individual variation in serum level 1 h after 8-MOP ingestion was lowest when the drug was taken during fasting conditions. On 12 of 20 occasions when 8-MOP was taken after a meal, the 1 h serum concentration was below 30 ng/ml. The frequency of a 1 h serum level below 30 ng/ml was significantly higher when the drug was taken after a meal than during fasting (Fisher's exact test, $P<0{\cdot}002$).

Nausea and dizziness occurred during 12 of the 30 8-MOP exposures. The frequency of side-effects was significantly increased both when C_{max} was above 400 ng/ml (Fisher's exact test, $P<0{\cdot}0005$), and when 8-MOP was taken during fasting conditions (Fisher's exact test, $P=0{\cdot}039$).

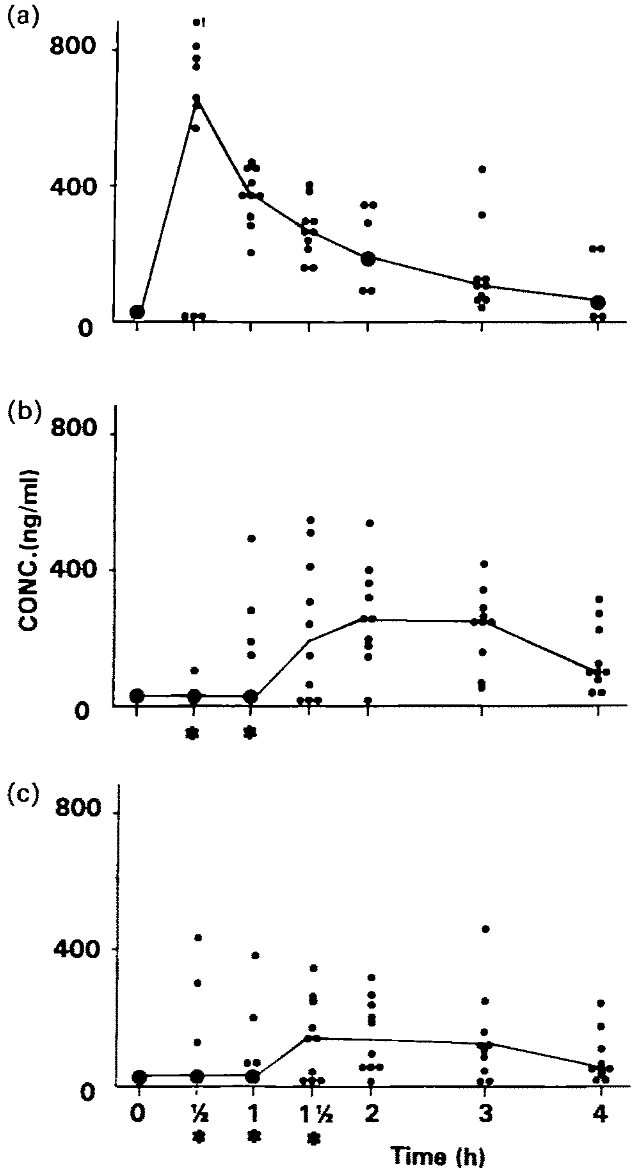

Figure 1. 8-MOP serum concentration, vs. time after drug ingestion in the 10 volunteers (a) Fasting conditions, (b) low-fat breakfast, (c) fat-rich breakfast. The solid line connects median values at each time point. *, significantly different from fasting •, one observation; ●, five–ten observations.

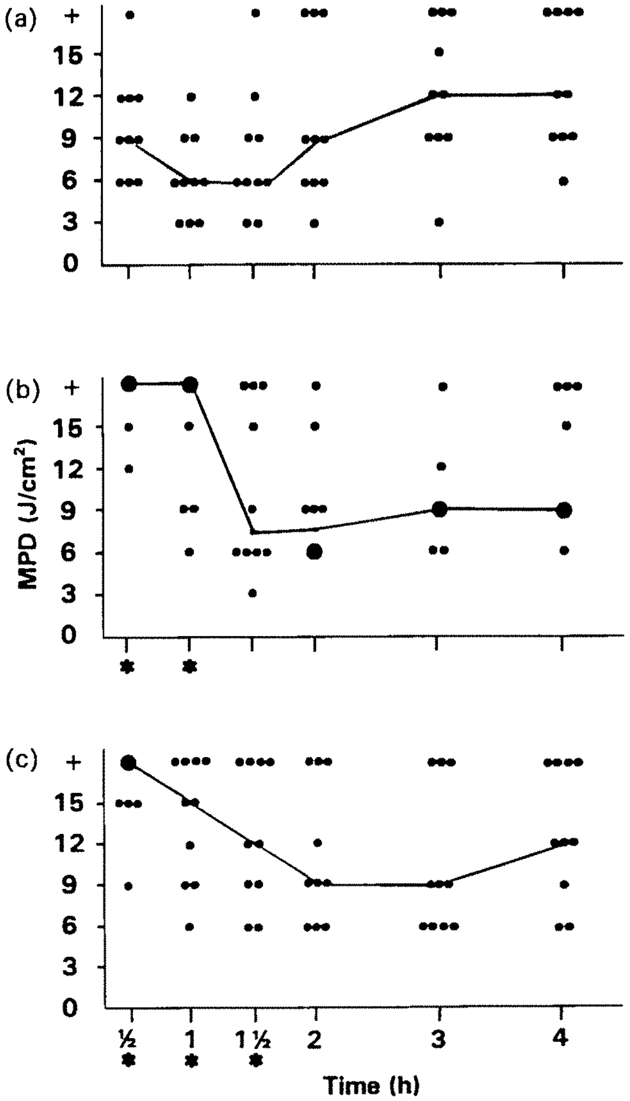

Figure 2. Minimal phototoxic dose (MPD), vs. time after drug ingestion for the 10 volunteers. (a) Fasting conditions, (b) low-fat breakfast, (c) fat-rich breakfast. The solid line connects median values at each time point. *, significantly different from fasting. •, one observation; ●, five–ten observations.

Photosensitivity

Photosensitivity was significantly more pronounced 0·5 and 1 h after drug intake during fasting than after both a low-fat and a fat-rich breakfast (Fig. 2). Time to the lowest MPD was significantly delayed by food intake, compared with fasting (median time 1·25 h) [$P<0·01$].

The intra-individual variation in photosensitivity (Fig. 3) was most pronounced 0·5 and 1 h after drug ingestion, when the UVA dose required to elicit erythema was on average 7·5 J/cm^2 higher after food than during fasting.

Out-patient survey

Thirty-seven out of a total of 1012 PUVA treatments resulted in erythemal reactions. Of the 43 out-patients studied, 22 did not develop erythemal reactions, 13 suffered one reaction during a PUVA course, three two, and five ≥3 reactions. The relationship between a >50% reduction in the usual quantity of food ingested by the patient before or with 8-MOP and the occurrence of erythemal reactions is shown in Table 1. The

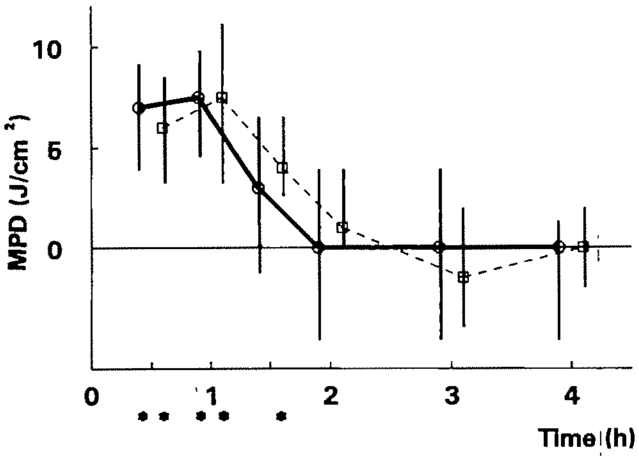

Figure 3. Intraindividual differences in minimal photoxic dose (MPD), vs. time after drug ingestion. (Median differences and 95·1% confidence intervals.) *, significantly different from fasting. —○—, low-fat and fasting; – –□– –, fat-rich and fasting.

frequency of adverse erythemal reactions was significantly higher when the quantity of food was >50% lower than the patients' usual intake (chi-square test; $P<0{\cdot}005$).

Of the 37 erythemal reactions, 25 occurred in the middle of the PUVA treatment course, where dose increments are about 10%, and seven occurred after the final level of UVA exposure was reached.

Discussion

Several investigators have studied the influence of the timing of food ingestion on 8-MOP kinetics. Most of the studies have shown a decrease in C_{max} and/or a delay in time to C_{max} when 8-MOP is taken with food.[4,5] In contrast, a single study found that food doubled C_{max} without influencing the time to C_{max}.[6] These differing results are probably related to differences in drug formulations and the composition of the diets. Our study showed a significantly decreased C_{max} and a delay in time to C_{max} when 8-MOP was taken after a meal. This signifies that the absorption of 8-MOP in a liquid formulation is significantly influenced by the presence of food. The results of our study contrast with an earlier study where no variations in serum levels, which could be attributed to food intake, were observed.[1] However, in the earlier study, there was no intra-individual comparison of the influence of food intake on 8-MOP serum levels, and the data were not processed statistically.[1] In agreement with some previous studies,[2,7] we did not find any significant influence of dietary fat content on 8-MOP absorption.

Table 1. Results of the out-patient survey (43 patients receiving a total of 1012 PUVA treatments)

Diet	Erythema	No erythema	Total	Frequency (%) of erythema with 95% confidence limits
>50% lower food intake than usual	8	13	21	38·1 (18·1–61 6)
Usual food intake	29	962	991	3·0 (2·0–4 3)
Total	37	975	1012	

The median intra-individual difference in MPD between fasting and post-prandial regimens in the 10 volunteers at 0·5 h and 1 h is probably larger than shown in Figure 3. After ingestion of either of the two breakfasts, many of the 15 J/cm² test sites in the volunteers did not show any sign of reaction. In these cases, an artificial photosensitivity value of 18 J/cm² was applied. It is likely that the real photosensitivity was considerably higher than the artificial value.[8]

In the clinic, UVA irradiation is administered 1 h after drug ingestion, when the influence of food on MPD is greatest (Fig. 3). The increased risk of adverse erythemal reactions is often reduced by using low increments in UVA dose, but this prolongs the PUVA course. This study shows that the intra-individual variations in MPD between fasting and post-prandial conditions are lowest 2 h after drug ingestion (Fig. 3). This means that the food-induced variations in photosensitivity can be reduced by delaying the time of UVA exposure to 2 h after drug ingestion. This is particularly preferable when the patients' dietary habits are not consistent.

It is recommended that 8-MOP is taken with food in order to counter adverse effects, such as nausea and dizziness, associated with ingestion of the drug.[9] In this study, we found that there was a greater correlation between high serum levels of 8-MOP and the occurrence of nausea and dizziness, than between the fasting state and these adverse effects. We observed very high 8-MOP serum levels when the drug was taken during fasting conditions. This suggests that therapeutic 8-MOP serum levels may be obtained during fasting by administering the drug in a dose lower than that currently recommended, which will probably reduce the occurrence of nausea, dizziness and drowsiness.

In conclusion, this study has shown, in contrast with previous opinions, that the absorption of 8-MOP in a liquid formulation is greatly influenced by the amount of food taken prior to drug ingestion. The fat content of the food does not influence the absorption of 8-MOP. In order to optimize the therapeutic effect of PUVA, it is necessary that the quantity of food taken before 8-MOP remains constant, and that the timing of UVA irradiation is adjusted according to the preceding food intake.

Acknowledgments

We are grateful for the nursing assistance provided by the staff at the Department of Dermatology at Copenhagen City Hospital, Bispebjerg. We thank Andreas Peterson for skilful technical assistance. This work was funded by Aage Bang's fond.

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British Journal of Dermatology (1992) 127, 625–629.

Therapy of alopecia areata with diphencyprone

E.HOTING AND A.BOEHM
Department of Dermatology, University Clinic Hamburg-Eppendorf, Hamburg, Germany

Accepted for publication 1 July 1992

Summary

The properties of diphencyprone (DPC), its possible mechanism of action in the therapy of alopecia areata (AA), and its side-effects are summarized. The results of our own study of treatment in 45 patients with AA, with a mean treatment duration of 72 (15–146) weeks, are presented, and compared with the results of previous studies. We suggest that, in future, therapy protocols should define entry criteria and therapy results more precisely, and include follow-up assessments to establish the length of remission. In view of the low response rates and short lengths of remission, the indications for DPC therapy should be set more rigorously.

The results of numerous investigations in recent years confirm the belief that autoimmune mechanisms are involved in the pathogenesis of AA.[1–3] Contact sensitizers, which are thought to act as immunomodulators in AA, have gained in popularity for the treatment of this disorder. Since the early 1980s diphencyprone (DPC) has been the preferred contact sensitizer for topical therapy of AA.[5–7] The eliciting of an allergic reaction after application of DPC appears to be an integral part of successful therapy.[8] Unwanted side-effects include a vesicular contact dermatitis, a generalized eczema, pigmentary changes, and nuchal lymphadenitis.[9–11]

In comparison with other contact sensitizers, DPC does not have the disadvantages of the instability in solution of squaric acid dibutylester (SADBE), or the mutagenic properties of dinitrochlorobenzene (DNCB).[12] However, mutagenic properties of a precursor of DPC and photomutagenic properties of DPC itself have recently been demonstrated.[13] To date, there are no reliable data available about the chronic toxicity and toxicokinetics of DPC.

In view of the necessary reaction accompanying therapy, the possible side-effects, and uncertainty about long-term toxicology, indications for DPC therapy should be clearly defined. A necessary prerequisite for the continued use of DPC in the treatment of AA is an assessment of its effectiveness, i.e. the expected mean response rate and length of remission. To this end, we evaluated DPC therapy in 45 patients and compared our results with those of other authors.

Methods

Subjects

The mean age of the patients ($n=45$; 21 male, 24 female) at the beginning of therapy was 32 (14–57) years. The presence of alopecia totalis or universalis, or the extension of AA over more than 75% of the scalp, without evidence of spontaneous remission, were prerequisites for the initiation of therapy. If the eyebrows were also affected, they were not treated, because of the risk of causing an allergic blepharoconjunctivitis by spread of DPC into the eyes.[14] Children, pregnant women and nursing mothers were excluded. Details elicited in the case history included the duration and course of the disease, and associated disorders such as atopy, vitiligo, and other autoimmune diseases.

Regimen

All patients were informed of the nature of the treatment and possible side-effects, and the uncertain long-term toxicology of DPC, and written consent was obtained. The patients were not allowed to administer their own treatment. Following initial sensitization with a 2% DPC solution, DPC was applied once a week to one side only, until hair regrowth on the treated side was clearly recognizable. In this way, it was possible to distinguish spontaneous remission, in which growth over the entire scalp would be expected. The concentration of the solution was increased or reduced depending on the side-effects. DPC dissolved in acetone was used in the following concentrations: 0·0001, 0·001, 0·01, 0·1, 0·25, 0·5, 1, 1·5, 2 and 3%. If there was no response, the treatment was discontinued after 20 applications. When

Correspondence: Dr E.Hoting, Univ. Hautklinik-Eppendorf, Martinistr. 52, D-2000 Hamburg 20, Germany

vellus hair regrew within this time period, the treatment was continued, but the regrowth of vellus hair alone was classified as a failure to respond to therapy. Regrowth of pigmented or non-pigmented terminal hair was judged to be a partial remission (PR) if it was necessary to wear a wig because of inadequate density, or gaps in the hair. Complete regrowth, with the exception of a single large bald patch which could be covered by adjacent long hair, was also scored as partial remission. According to our standards, a complete remission (CR) was present when all hair regrew, or at the most only two/three bald patches of no more than 1–2 cm in diameter remained, making the wearing of a wig unnecessary.

Table 1. Results of DPC therapy in patients with AA

Patient data	
No. of patients	$n=45$
M/F	21/24
Age at beginning of therapy (years)	$x=32$
(range)	(14–57)
Age at first manifestation <15 years	$x=17$
Total duration of disease (years)	$x=9{\cdot}4$
(range)	(0·1–52)
Duration of therapy (weeks)	$x=72$
(range)	(14–176)
Form of alopecia	
Universalis	$n=14$ (31%)
Totalis	$n=20$ (44%)
Ophiasis	$n=7$ (15%)
Large focus AA (>75%)	$n=4$ (9%)
Family history	
Atopy	$n=9$ (20%)
Atopic dermatitis	$n=1$ (2%)
Alopecia areata	$n=2$ (4%)
Associated diseases	
Atopy	$n=9$ (20%)
Atopic dermatitis	$n=4$ (9%)
Vitiligo	$n=2$ (4%)
Goitre	$n=2$ (4%)
Response rate	
Complete remission	$n=9$ (20%)
Partial remission	$n=14$ (31%)
Relapse rate (total)	$n=15$ (33%)
Relapse during therapy	$n=8$ (18%)
No response	$n=22$ (49%)
Side-effects	
Severe dermatitis	$n=20$ (44%)
Spreading of foci	$n=3$ (7%)
Hyperpigmentation	$n=1$ (2%)
Lymph node swelling	$n=14$ (31%)

Results

Fourteen of the 45 patients had alopecia universalis, 20 alopecia totalis, and 11 had alopecia of the ophiasis type, or a multicentric, large focus AA (Table 1). In 17 of the patients with alopecia totalis, multicentric AA or ophiasis, eyebrows, eyelashes and beard hair were also affected. The first manifestation occurred at an age of less than 15 years in a total of 17 patients. The total duration of the disease prior to starting DPC therapy averaged 9·4 (0·1–52) years. Half the patients had experienced an episode of disease activity during the year preceding therapy. A concomitant atopic diathesis was present in 18 (40%) patients, and four (9%) had manifest atopic dermatitis. The family histories suggested a possible atopic diathesis in 10 (22%) of the cases. In two (4%) of the patients the parents or grandparents had suffered from AA. Vitiligo was present in two (4%) of the patients and a euthyroid goitre in two (4%) of the cases (Table 1).

The mean duration of therapy was 72 (14–146) weeks. The total observation period, including follow-up examination for which 21 patients attended, averaged 2·6 years (=136 weeks).

The first regrowth of hair appeared on average 17 (4–32) weeks after the start of treatment. Altogether 23 (51%) patients responded to therapy; nine (20%) obtained a CR and 14 (31%) a PR. Of the nine patients with a CR, four suffered a relapse during therapy and five a relapse after the end of therapy. The length of remission after stopping treatment was 1–24 months (2 × 1, 1 × 5, 1 × 12, 1 × 24 months). In four patients it was possible to maintain a PR after a relapse had occurred by continuation of therapy. Likewise, of the 14 patients with a PR, four suffered a relapse during therapy, and six after stopping therapy. The length of remission varied from 3 months to 5 years (1 × 1, 1 × 3, 1 × 6 months, 1 × 1, 1 × 4, 1 × 5 years). Four patients defaulted without explanation (Table 1).

Twenty-two (49%) patients in whom no hair growth was apparent, or only thin, non-pigmented vellus hair regrew, were counted as failing to respond to therapy. Thirteen non-responders were from the group of patients with alopecia universalis ($n=14$). Of the 18 patients with a total disease duration exceeding 10 years, five responded to therapy (5 × PR), whereas of the 17 patients with a short disease duration (<1 year), 10 responded to therapy (5 × CR, 5 × PR) [Table 1].

Table 2. Survey of results of DPC therapy

Reference	No of patients	Form of alopecia	Length of therapy (weeks)	Response rate	CR+PR (%)	Relapse rate
Kietzmann *et al.*, 1985[17]	20	7 universalis 1 totalis 12 AA>25%	20	12 CR 5 PR	85	?
Ochsendorf *et al.*, 1987[27]	27	1 universalis 8 totalis 8 AA >75% 8 AA <50%	20–?	10 CR 11 PR	77	10 during therapy
Happle *et al.*, 1984[16]	34	25 totalis 9 extensive AA	16–68		70	?
Orrecchia, Rabbiosi 1985[28]	26	10 universalis 7 totalis 9 patchy AA	16–60	1 CR 13 PR	53	4 during therapy
MacDonald-Hull, Norris 1984[29]	28	13 totalis 11 AA >50–75% 4 AA <50%	32	8 CR 4 PR	42	?
Monk, Williams 1988[15]	15	13 totalis 2 patchy AA	52	6 PR	40	10 during therapy
Ashworth *et al.*, 1989[12]	17	9 universalis 8 totalis	20–30	1 CR 2 PR	17	?
Hoting and Boehm (present study)	45	14 universalis 20 totalis 11 AA >75%	14–146	9 CR 14 PR	51	19 total, 8 during therapy

CR, complete remission; PR, partial remission.

Discussion

While Ashworth *et al.*[12] noted hair regrowth in only 17% of patients, Monk and Williams[15] reported 40%, Happle *et al.*[16] 70% and Kietzmann *et al.*[17] 85% regrowth (Table 2). Our own response rate was 51%. How can such differences be explained when the substance is applied using identical methodology?

1. *Entry criteria*

The response rate is markedly influenced by the criteria for entry into therapy. Prognostically unfavourable features in the long-term are extensive involvement, disease of long duration and the presence of an atopic diathesis, atopic eczema, or associated autoimmune diseases. A study should therefore always contain an exact description of the patient group, and a definition of the entry criteria. None of the studies listed in Table 2 completely fulfilled these requirements. Only the extent of the involved area was stated by all authors. In accordance with the unfavourable prognosis accompanying extensive hair loss, Ashworth *et al.*[12] obtained a response rate of only 17% when patients with alopecia totalis or universalis were treated, in contrast with a response rate of 77% or 85% observed by authors whose patient groups included a large number of subjects with small focus AA.[16,17]

When excellent responses to treatment are reported, the suspicion that spontaneous cures may have influenced the results always arises. In view of the favourable prognosis and high spontaneous cure rates in approximately 30% of cases of small focus AA with involvement of less than 40% of the scalp, some therapeutic measures, including topical DPC, may be difficult to justify.

2. *Disease duration before initiation of therapy*

According to the observations of Happle *et al.*[16] the duration of the disease is apparently without influence on the success of DPC therapy. However, MacDonald Hull *et al.*[18] attributed their markedly lower response rate of 42% to the fact that only patients with a long disease duration (average 19·9 years) were recruited. In our group only five out of 18 patients with a total disease duration of more than 10 years responded to treatment, in comparison with 10 of 17 patients with a disease duration of less than 1 year.

3. *Definition of complete and partial remission*

The differing and somewhat inexact definition of the terms CR, PR and failure to respond to therapy contribute to the strongly contrasting results.

According to our criteria, a CR was present when the hair had fully regrown, or a maximum of three bald patches, no larger than 1 cm in diameter, remained. Some other authors did not employ similar exact limits.[8,19] In contrast with other authors, we considered a partial remission to be present when a single large focus could be covered by the remaining hair, or more than three foci with a diameter larger than 1 cm were present. We scored the regrowth of vellus hair alone as a failure to respond to therapy. In contrast, MacDonald Hull *et al.*[18] evaluated vellus hair as the weakest form of response. In other studies it is not evident whether, or how, the regrowth of vellus hair was classified.

In an appraisal of the value of DPC therapy in the treatment of AA, the duration of remission must be included, in addition to the response rate. Hence, of 23 of our patients who responded to therapy, eight (35%) had already suffered a relapse during treatment, and a further eight (35%) suffered a relapse within the first year after stopping therapy. In some other reports, details concerning the duration of remission are completely absent.

In order to assess the value of DPC treatment in AA, a comparison with other treatment methods should be made. In addition to other contact sensitizers such as SADBE and DNCB, treatments for AA include topical and systemic corticosteroids, PUVA therapy, topical cyclosporin, topical minoxidil, and the immunomodulator inosiplex. However, all these therapies either have significant side-effects, or a real benefit is hardly demonstrable.[20–25]

In conclusion, we consider that the indications for DPC therapy should be set more rigorously, in view of the relatively low response rates and short remission times, and because of possible long-term risks (photomutagenesis, uncertain long-term toxicology). Children, pregnant women and nursing mothers should not be treated. Therapy should be limited to patients with extensive forms of AA involving more than 75% of the scalp in whom the disorder is causing a significant degree of suffering. In future, therapy protocols should precisely define not only entry criteria, but also therapy outcome, and include follow-up examination to assess the length of remission, as has also been proposed by other authors.[3,26] For these reasons, we suggest that DPC therapy should be carried out in specialized centres, in order to further clarify its role in the treatment of alopecia areata.

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British Journal of Dermatology (1992) 127, 630–634.

Restrictive dermopathy: a disorder of fibroblasts

D.G.PAIGE, B.D.LAKE,* A.J.BAILEY,† P.RAMANI* AND J.I.HARPER

*Departments of Dermatology and *Histopathology, The Hospital for Sick Children, Great Ormond Street, London U.K.*
†Muscle and Collagen Research Group, University of Bristol, Langford, Bristol, U.K.

Accepted for publication 8 July 1992

Summary

Restrictive dermopathy is a rare, lethal genodermatosis, characterized by a thin, tightly adherent skin which causes a dysmorphic facies, arthrogryposis and respiratory insufficiency. The recorded cases to date show a remarkable phenotypic similarity. Thinning of the dermis and the arrangement of collagen in parallel bundles appear to be constant findings. We have found many dead and degenerating fibroblasts in the dermis on ultrastructural examination, and have demonstrated their poor growth *in vitro*. Studies of collagen from a skin sample showed a marked increase in mature cross-links, indicating a decrease in skin collagen turnover. These findings suggest a primary disorder of fibroblasts, and may explain the apparent arrest in growth and differentiation of the skin which appears to be important in the pathogenesis of this rare condition.

Case report

A female baby was born at 31 weeks' gestation by lower-segment Caesarean section. This was performed as an emergency procedure as the baby was noted to have a transverse lie and signs of fetal distress following premature rupture of membranes. The previous course of the pregnancy had been uncomplicated except for a minor episode of vaginal bleeding at 10 weeks. The Caesarean section was carried out with some difficulty, and the baby sustained a tear to the skin of the right arm. Birth weight was 1·44 kg. A large placenta weighing 850 g was delivered (expected normal weight 300–400 g). The baby was noted to have low Apgar scores at birth, and poor respiratory effort. She was artificially ventilated with good effect, and after a few hours managed satisfactorily with a head box and 40% oxygen.

The parents were Caucasian and were non-consanguineous. There had been two previous pregnancies. The first resulted in a normal, term baby. The second ended in a miscarriage at 27 weeks; subsequent post-mortem examination was reported as showing no abnormality, although the skin was not specifically examined.

On examination at birth, the child was noted to be dysmorphic (Fig. 1a–c). There was a large, soft anterior fontanelle with splayed sutures, low-set ears, hypertelorism and micrognathia. The eyes were slanted in an anti-mongoloid fashion, and the nose and mouth were small. The skin was thin and tightly bound down, producing joint contractures at the elbows, fingers, wrists, knees and hips. The skin blood vessels were prominent, consistent with the baby's prematurity. A laceration was noted on the right arm. In spite of assisted ventilation, the baby died of respiratory failure at the age of 3 weeks.

Investigations

Chromosome studies showed a normal 46 XX karyotype. A chest X-ray revealed poorly inflated lungs, and abnormal bone modelling at the proximal ends of both humeri. There were marked osteodysplastic changes in the clavicles (Fig. 2) and an ossification defect of the skull vault was noted.

Two 4-mm skin punch biopsies were taken 2 days after birth for light and electron microscopy, collagen cross-linking analysis and fibroblast culture. Light and electron microscopy were performed on tissue prepared as previously described.[1] Mild parakeratosis and hyperkeratosis were noted in the epidermis. Light microscopy showed marked thinning of the dermis, and the collagen fibres were arranged in bundles parallel to the skin surface (Fig. 3a). Elastic tissue was present in normal amounts. Eccrine sweat glands and a few sebaceous glands were seen. Both immature and mature hair follicles were present, but adnexal structures were generally decreased in number. The dermo-epidermal

Correspondence: Dr J.I.Harper, Consultant Dermatologist, The Hospital for Sick Children, Great Ormond Street, London WC1N 3JH, U.K.

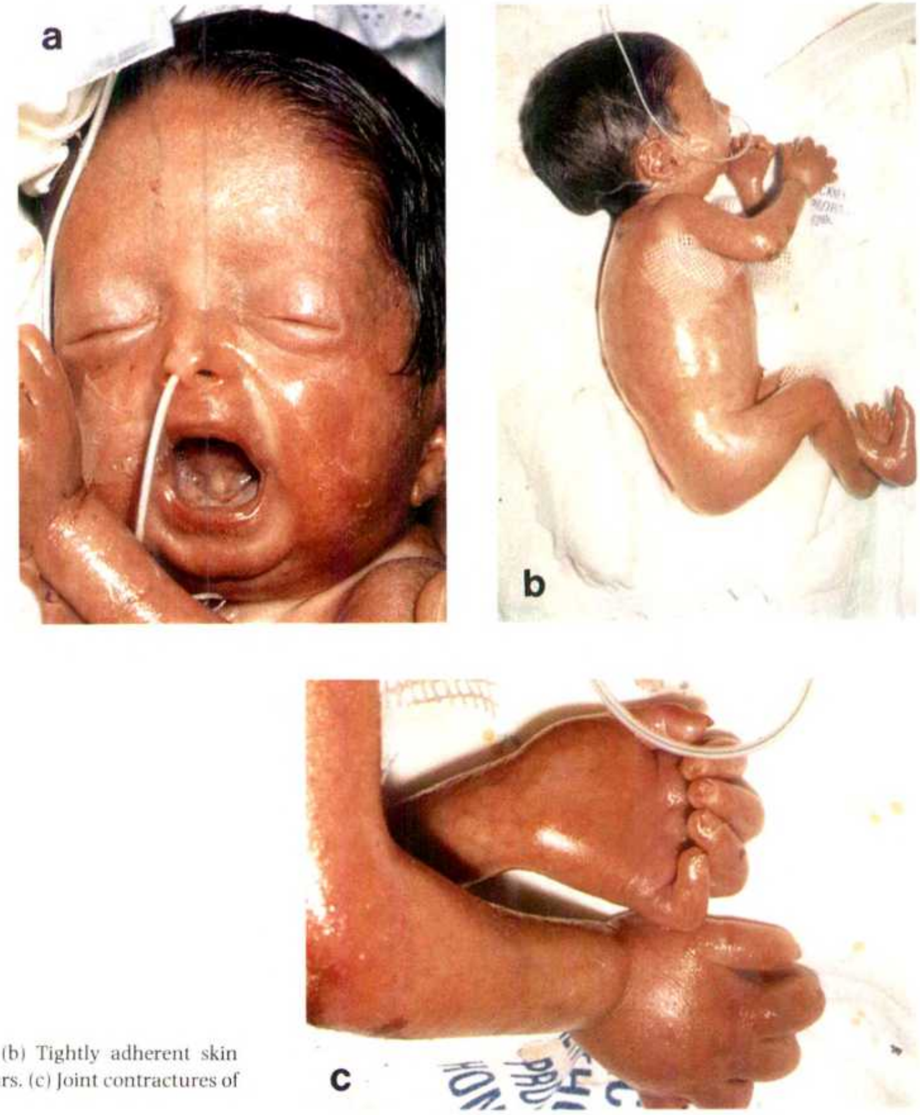

Figure 1. (a) Typical dysmorphic facies. (b) Tightly adherent skin causing joint contractures and deformed ears. (c) Joint contractures of the hands.

junction was flattened, and the subcutaneous tissue appeared normal, with no fibrous tissue framework. Similar light microscopy findings were seen at three other skin sites biopsied at post-mortem. Immunostaining using antibody AE1 (Ortho Diagnostic Systems Ltd, High Wycombe, U.K.) showed positive reactivity only in the sweat glands (Fig. 3b), with no reactivity in any of the epidermal layers.

Electron microscopy showed no significant abnormalities in the epidermis, and the basement membrane structure was normal. In the dermis, collagen fibrils were of normal size. Elastic tissue and microfilaments were present, and did not appear reduced in amount. Blood vessels and unmyelinated nerve fibres were present and of normal structure. The most significant abnormality was in the fibroblasts, many of which appeared to be degenerating, although an occasional active fibroblast with dilated rough endoplasmic reticulum was seen (Fig. 4a and b). The fibroblasts initially grew very slowly in culture, then entered a quiescent phase, surviving in this state for several weeks. During this time, the growth of cells could not be stimulated by the use of enriched media from IMR 90 cells or from a secondary human skin line, or by the use of collagen gels. The fibroblasts did not synthesize collagen *in vitro*, and attempts at trypsinization led to cell death.

Skin biopsies were analysed for the intermolecular cross-links stabilizing the collagen fibres (Table 1). Analysis of the collagen fibres following borohydride reduction, acid hydrolysis, and identification of the cross-links on an LKB AutoAnalyser[2] revealed the presence of intermediate divalent cross-links, hydroxy-

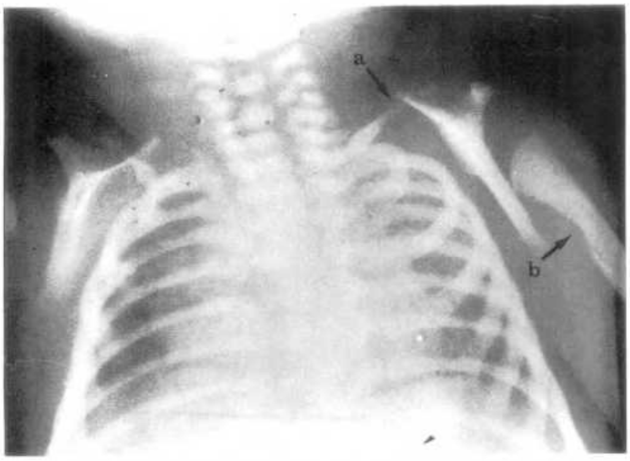

Figure 2. Chest X-ray showing osteodysplasia of the clavicles (a) and (b) modelling defects of the proximal humeri.

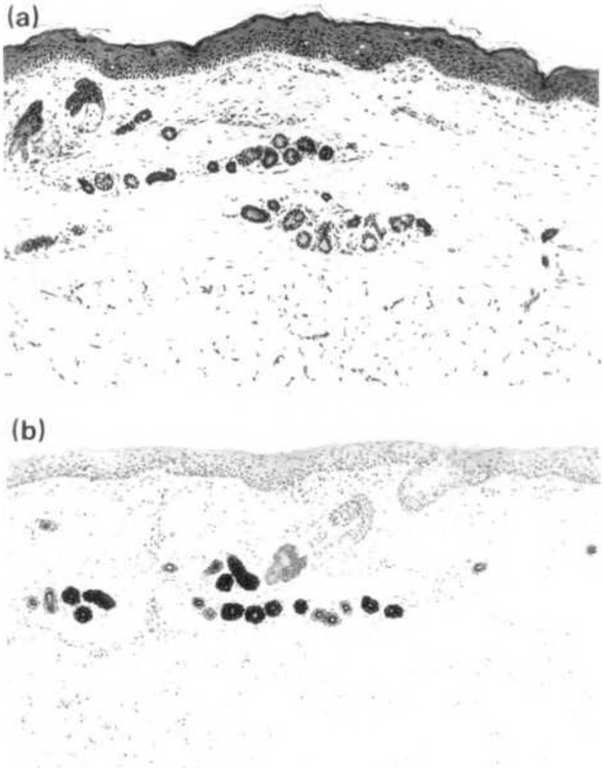

Figure 3. Skin biopsy showing (a) thin dermis with horizontally aligned collagen fibres. Sweat glands are also aligned in the same plane. The ratio of the thickness of epidermis to dermis is about 1 : 5, in contrast with the normal of 1 : 10–1 : 20 at this age (haematoxylin and eosin × 90). (b) Immunoreactivity with antibody AE1 confined to the sweat gland epithelium. No immunoreactivity was found in any of the epidermal cell layers. (ABC peroxidase with a weak haematoxylin counterstain × 90.)

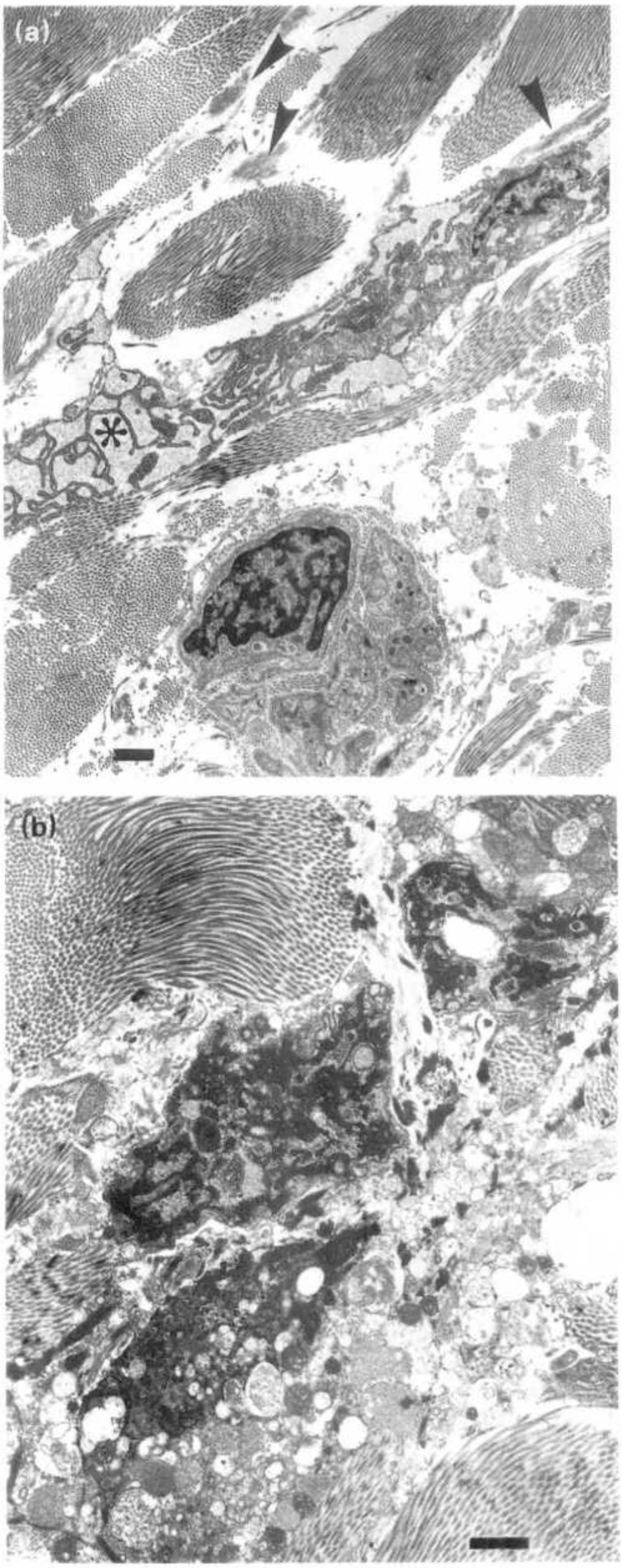

Figure 4. Electron micrograph showing (a) a fibroblast with dilated endoplasmic reticulum (*) and a normal Schwann cell and nerve fibres (centre bottom). Elastic fibres are present (arrows) (× 4800; scale bar = 1 μm). (b) Three degenerate fibroblasts (× 7300; scale bar = 1 μm).

Table 1. Collagen cross-links present in the dermis

Cross-link	m/m collagen	Ratio of cross-links
DHLNL	0·10	
HLNL	0·31	HLNL/DHLNL 3:1
HHL	0·07	HLNL/HHL 4:1 (normally trace of HHL)
PYR	0·05	DHLNL/PYR 2:1

DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; HHL, histidino-hydroxylysinonorleucine; PYR, hydroxylysinopyridinoline.

lysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL) in a ratio of 3:1, as expected for skin collagen at this age. In addition, we identified a surprisingly high proportion of the mature trivalent cross-link histidino-hydroxylysinonorleucine (HHL) relative to its precursor HLNL (ratio HLNL:HHL 4:1). Normally, in immature collagen, the cross-link HHL is barely detectable. A small proportion of hydroxylysyl-pyridinoline (OH-PYR) was also detected (ratio DHLNL:OH-PYR 3:1).

At post-mortem the internal organs appeared macroscopically and histologically normal, although the bones were not formally examined. The lungs were not hypoplastic and there was no evidence of calcification in the skin or internal organs.

Discussion

Restrictive dermopathy was first recognized as a diagnostic entity in 1986 by Witt *et al.*[3], who described a lethal condition in two premature siblings from consecutive pregnancies. They suffered from tightly adherent skin in association with generalized flexion contractures, pulmonary hypoplasia and characteristic facies, and they had abnormally large placentas. Probably the earliest recorded cases were reported by Leschot *et al.*[4] (1980) and Toriello[5] (1983) under the diagnostic category of 'aplasia cutis congenita'. The cases of unusual 'congenital contractures' reported by Lowry *et al.*[6] may also have been examples of restrictive dermopathy, although only minimal dermal changes were described on skin biopsy. The two cases of Carmi *et al.*[7] had some phenotypic similarities, but they also suffered from large areas of denuded skin, and had somewhat atypical skin histology. The presence of pyloric atresia in one of these patients raises the possibility of junctional epidermolysis bullosa as the diagnosis. A case of 'a lethal ichthyosis variant with arthrogryposis' described by Schur *et al*[8] has also been called restrictive dermopathy, but we feel that the reported clinical appearance and skin histology are more in keeping with a collodion baby progressing to lamellar ichthyosis.

To date, at least 17 definite cases of restrictive dermopathy have been reported in the literature.[3-5,9-14] The clinical features of these cases have recently been reviewed.[13] The condition is thought to be inherited as an autosomal recessive trait. The phenotypic appearance of these babies is strikingly similar. Most are born prematurely between 30 and 33 weeks gestation. Osteodysplasia of the clavicles with modelling defects of the long bones would also appear to be a common feature of this universally lethal condition. Most infants die shortly after birth from respiratory failure. As yet, there is no reliable prenatal diagnostic test. Fetal skin biopsy has been carried out at the 20th week of gestation on a boy who was subsequently found to have the condition at birth, but this revealed no abnormality on light or electron microscopy.[14] However, no specific comment was made on the state of the dermal fibroblasts.

The cause of this rare genodermatosis is, as yet, unknown. A variety of epidermal changes have been noted.[10,12-14] Mild hyperkeratosis and parakeratosis are frequent findings on light microscopy. Electron microscopy has shown widely spaced basal cells. Keratohyaline granules are sometimes seen to be patchy in distribution and globular in shape, and they lose their association with keratin filaments. Vacuolization in the stratum corneum and a decrease in keratin filaments has also been reported. Biochemical studies have revealed the presence of 48- and 56-kDa keratins suggestive of hyperproliferation, further supported by a suprabasal staining pattern with the anti-keratin antibody AE1.[10] However, we were unable to confirm this staining pattern.

The dermo-epidermal junction appears flat, but is normal in structure. The subcutaneous tissue has been described as thickened, and in some cases has a framework of fibrous connective tissue.[10,11] A decrease[10,13] or absence[12,14] of elastic tissue has also been reported in those cases where ultrastructural studies were performed.

It is our opinion that the changes in the epidermis and subcutaneous tissue are of minor importance. They are inconstant, and probably reflect changes secondary to dermal pathology, because the dermis is known to have considerable influence over epidermal differentiation.[15-18] The changes of dermal thinning and the parallel alignment of the collagen fibres appear to be a constant feature in the recorded cases. We feel that the primary problem is most likely to lie within the dermis, and have demonstrated the presence of markedly abnormal fibroblasts. Although in some instances these appear to be active and secretory, many fibroblasts also appear degenerate. Interestingly, we have also found that fibroblasts from this patient showed poor growth *in vitro*, and did not produce collagen. The stability and turnover of the skin collagen was assessed by analysis of the intermolecular cross-links. Under normal development, the collagen is turning over relatively rapidly and is stabilized primarily by the intermediate cross-link dehydro-hydroxylysinonorleucine, but as turnover decreases the collagen matures and the cross-link is converted to the mature trivalent cross-link, histidino-hydroxylysinonorleucine.[19] Our finding of a high proportion of the latter in the skin collagen of this baby clearly indicates an abnormally mature state of the skin collagen, and implies a profound decrease or complete arrest in collagen turnover. It would appear that the fibroblasts have been 'switched off' in the skin. The presence of osteodysplasia, which is a common finding in this condition, may reflect a more widespread mesenchymal disorder.

We propose that restrictive dermopathy is a genetically determined disorder of fibroblasts. This causes an arrest in the normal development of the dermis, probably in the second trimester, and continued fetal growth within a 'static' dermis causes the distinctive clinical picture and histological appearance.

Acknowledgments

We thank Dr K.Costelloe (consultant paediatrician, The Homerton Hospital, London) for referring the patient, Dr P.C.Conn (consultant histopathologist, Severalls Hospital, Colchester) for providing post-mortem tissue, and S.Geddes (Division of Biochemistry, Institute of Child Health, University of London) and V.Smith (Department of Histopathology, Hospitals for Sick Children, Great Ormond Street, London) for technical assistance.

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British Journal of Dermatology (1992) 127, 635–640.

Cytophagic histiocytic panniculitis: a report of four cases

T.PETTERSSON, A.-L.KARINIEMI, S.TERVONEN* AND K.FRANSSILA
Fourth Department of Medicine, Department of Dermatology and Department of Oncology and Radiotherapy, Helsinki University Central Hospital, Helsinki, Finland
**Department of Internal Medicine, Kuopio University Central Hospital, Kuopio, Finland*

Accepted for publication 23 June 1992

Summary Four patients presented with a clinical picture of spiking fever, erythematous nodular subcutaneous skin lesions, anaemia and leucopenia. Inconstant features were weight loss (3/4), splenomegaly (3/4), thrombocytopenia (3/4), raised liver enzymes (3/4), hepatomegaly (2/4) and serosal effusions (2/4). The histopathological findings of lobular histiocytic panniculitis with 'bean-bag' cells were characteristic of cytophagic histiocytic panniculitis (CHP). No infectious agent could be detected, and there was no histological evidence of malignant neoplasia. Unlike most previously described patients with CHP, three of our four patients responded to treatment with immunosuppressive or cytostatic drugs.

Cytophagic histiocytic panniculitis (CHP) is a histiocytic proliferative disorder originally described in 1980.[1,2] Morphologically benign histiocytes infiltrate adipose tissue, producing necrosis with oedema and haemorrhage. Despite the benign appearance of the histiocytes the disease is considered biologically malignant. It usually runs a chronic, febrile course with pancytopenia, mucosal ulceration, serositis and hepatosplenomegaly, and terminates in liver failure and death from haemorrhage.[1,2] CHP has been observed in association with infections and haematopoietic disease,[3] and recent evidence suggests that CHP may be a manifestation of clonal T-cell proliferation.[4]

We report four patients with a clinical picture and histopathological findings characteristic of CHP. Although all our patients were seriously ill at the time of diagnosis, remission was achieved by the use of either immunosuppressive or cytostatic therapy.

Case reports

Brief, preliminary reports of Cases 1 and 2 have been published previously.[5,6]

Case 1

A 61-year-old woman was seen in January 1978 because of fever of 4 months' duration (temperature up to 38·8°C) and a violaceous, tender plaque on her back (Fig. 1). There was no lymphadenopathy or hepatosplenomegaly. She had moderate anaemia, leucopenia ($1{\cdot}7\times10^9$/l) and an erythrocyte sedimentation rate (ESR) of 35 mm/h. Routine chemistry and chest X-ray were normal. Tests for antinuclear antibodies and rheumatoid factor were negative. A bone-marrow aspirate was normal. A biopsy of the skin lesion revealed a non-specific lobular panniculitis with fat necrosis, and histiocytes which had phagocytosed erythrocytes and leucocytes (Fig. 2). Extensive microbiological investigations failed to detect any infectious agent, and neither the fever nor the subcutaneous plaque were influenced by broad-spectrum antibiotics. Lymphangiography was normal.

During the following months her general condition deteriorated and she lost 20 kg in weight. New nodules appeared on her trunk and left arm. Her haemoglobin was 11 g/dl, leucocyte count $2{\cdot}5\times10^9$/l (with a neutrophil left shift), platelet count 104×10^9/l and ESR 7 mm/h. The level of aspartate aminotransferase (ASAT) was five times the normal level, that of alanine aminotransferase (ALAT) marginally increased, and that of alkaline phosphatase four times the normal level. An ultrasound examination of her abdomen revealed splenomegaly. A

Correspondence: Dr T.Pettersson, Fourth Department of Medicine, Helsinki University Central Hospital, Unioninkatu 38, SF-00170 Helsinki, Finland.

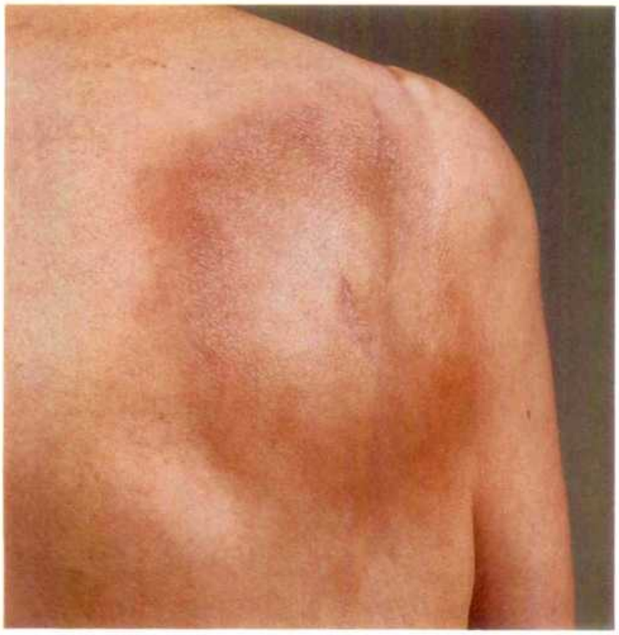

Figure 1. A tender, violaceous subcutaneous plaque on the back of Case 1.

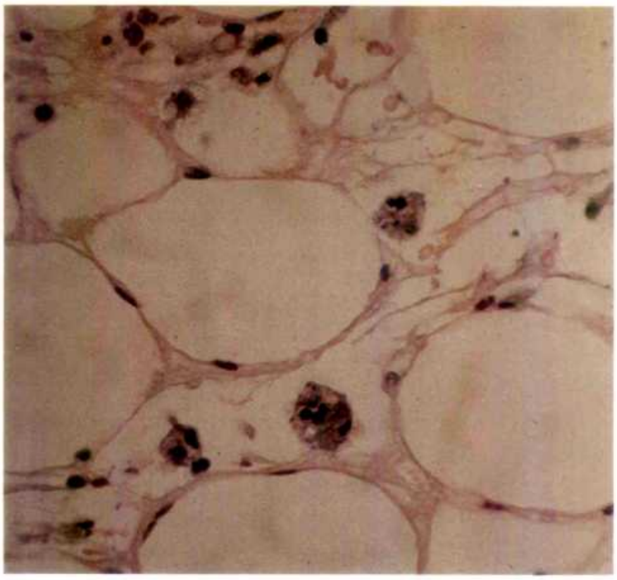

Figure 2. Characteristic 'bean-bag' cells, i.e. macrophages exhibiting erythro- and leucophagocytosis, in subcutaneous adipose tissue (haematoxylin and eosin × 400).

liver biopsy demonstrated fatty infiltration. A bone-marrow aspirate contained 10% histiocytes, some of which showed haemophagocytosis. She was considered to be suffering from histiocytosis, and Bleo-CHOP therapy was initiated (bleomycin 4 mg/m^2 and vincristine 2 mg, days 1 and 5; cyclophosphamide 750 mg/m^2 and doxorubicin 50 mg/m^2, day 1; prednisone 100 mg, days 1–5). Both the fever and the skin lesions responded favourably, but she developed severe leucopenia and died of pneumonia in December 1978, 2 weeks after the second course of Bleo-CHOP. Post-mortem revealed no histiocytic infiltration in any organ.

Case 2

A 23-year-old man was admitted to hospital in August 1987 because of recurrent fever (temperature up to 40·3°C) and tender, violaceous or brownish subcutaneous nodules on his face, trunk, arms and legs. No mucosal lesions or lymphadenopathy were detected, but the spleen was enlarged. He had leucopenia ($2{\cdot}8 \times 10^9$/l) but the haemoglobin, ESR, serum C-reactive protein (CRP) concentration, routine chemistry and chest X-ray were normal. Biopsy of a skin lesion showed a lobular panniculitis with fat necrosis and numerous histiocytes, which had phagocytosed erythrocytes and leucocytes.

A few weeks later he had developed anaemia (Hb 10·9 g/dl) and marginal thrombocytopenia (148×10^9/l), and the leucocyte count had decreased to $1{\cdot}6 \times 10^9$/l. The differential count and the ESR and CRP values remained normal. No rheumatoid factor or antinuclear antibodies were detected and concentrations of IgG, IgA and IgM, and complement components C3 and C4 were normal. The serum angiotensin converting enzyme (ACE) level was high (176 U/l, normal range 35–155 U/l) as was the serum lysozyme level (45 mg/l, normal range 8–17 mg/l). Extensive microbiological and serological investigations were negative for bacteria, fungi and parasites, and no significant titres of antiviral antibodies were detected, including antibodies to hepatitis A and B viruses, cytomegalovirus, Epstein–Barr virus and human immunodeficiency virus. Serum concentration of tumour necrosis factor-α (TNF-α) was determined with a radioimmunological technique and raised levels were found in serial samples (20–190 ng/l, normal range 0–40 ng/l).[7] Serum interleukin 1β (IL-1β) levels remained within the normal range. Computed scans of the thorax and abdomen were normal, except for the enlarged spleen. Bone-marrow aspirate and biopsy showed no histiocytic infiltration. A fine-needle aspirate of the spleen showed numerous histiocytes, which appeared benign (Fig. 3).

Treatment with antipyretic drugs, broad-spectrum antibiotics and prednisone 60 mg/day was unsuccessful. Splenectomy was therefore performed. The spleen weighed 675 g, and histology revealed histiocytic proliferation with haemophagocytosis, but no neoplastic

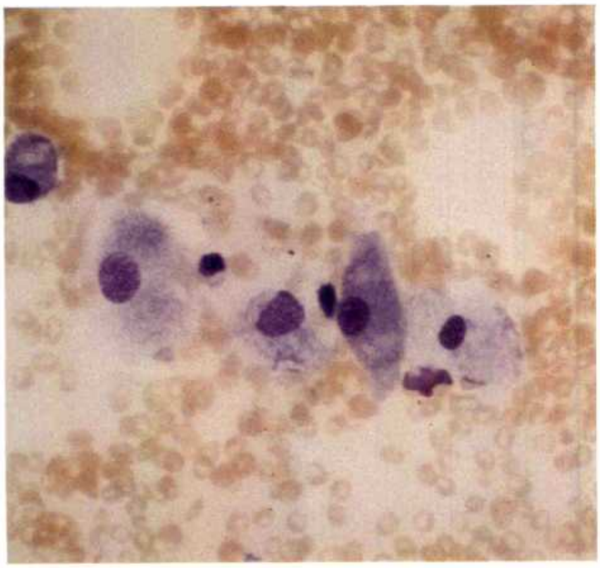

Figure 3. Benign-appearing splenic histiocytes in a fine needle aspirate (Giemsa × 1000).

features. A liver biopsy demonstrated fatty infiltration. A short course of antituberculous drugs and an intravenous infusion of cyclophosphamide (2 g) did not affect his symptoms. He had suffered a weight loss of 25 kg, and his general condition had deteriorated. He became jaundiced, and there was a marked increase in liver enzymes: ASAT rose to 338 U/l (normal range 8–36 U/l), ALAT to 417 U/l (8–40 U/l), alkaline phosphatase to 1180 U/l (60–280 U/l) and γ-glutamyl transpeptidase to 299 U/l (3–46 U/l). Serum concentration of lactate dehydrogenase (LDH) was 4320 U/l (normal <430 U/l). He had three bouts of acute pancreatitis, suffered from transient double vision, and developed peroneal paresis in both legs. Five months after admission, treatment with Bleo-CHOP was started, using the same dose schedule as in Case 1. After three Bleo-CHOP cycles (repeated every 3 weeks) his fever and skin lesions had disappeared. Cytostatic treatment was stopped, and $4\frac{1}{2}$ years later he remains in remission.

Case 3

A 17-year-old man was admitted to hospital in February 1989 because of recurrent fever, tender subcutaneous nodules, and weight loss of 5 kg. Initial investigations had shown anaemia, leucopenia ($1{\cdot}9 \times 10^9$/l) with a neutrophil left shift, and normal ESR and CRP values. Ultrasound examination of the abdomen had demonstrated mild hepatosplenomegaly and ascites. Bone-marrow aspirate and biopsy were normal, and a biopsy of a skin lesion showed non-specific panniculitis. There was no response to broad-spectrum antibiotics.

On admission his temperature was 41·0°C, and he had tender subcutaneous nodules on the abdomen and legs. The haemoglobin decreased to 8·3 g/dl, the leucocyte count was $2{\cdot}5 \times 10^9$/l and platelets 125×10^9/l. ESR and CRP remained normal. ASAT rose to 91 U/l, ALAT to 77 U/l and LDH to 2210 U/l. Serum lysozyme concentration was 8 mg/l (normal range 2–8 mg/l). Chest X-ray and serum IgG, IgA, IgM, C3 and C4 were normal. Extensive microbiological and serological investigations revealed no evidence of any infectious agent. Computed scans of the thorax and abdomen were normal apart from mild hepatosplenomegaly. Histology of a liver biopsy, taken at laparoscopy, was normal.

Two weeks after admission, therapy with prednisone, 60 mg/day, was started. His temperature returned to normal within 24 h. One month later, when the prednisone dose had been reduced to 15 mg/day, the fever and subcutaneous nodules recurred. A bone-marrow aspirate showed a mild increase in the number of mononuclear phagocytes, some of which exhibited haemophagocytosis. A fine-needle aspiration of the spleen was complicated by haemorrhage, which necessitated emergency splenectomy. Examination of the aspirate and the splenic tissue revealed numerous benign-appearing histiocytes exhibiting haemophagocytosis. Re-evaluation of the skin biopsies also revealed macrophages with phagocytosed red and white blood cells. He was successfully treated with prednisone 30 mg/day. Prednisone therapy was gradually tapered, and there have been no signs of relapse.

Case 4

A 20-year-old woman had an acute illness with fever, nausea, vomiting, diarrhoea and a swollen cheek during a journey to Thailand in March 1989. Two weeks later, when she had returned to Finland, tender, reddish subcutaneous nodules appeared on the abdomen and on her arms and legs. The fever persisted, she complained of headache, and had transient loss of vision in her right eye. Investigations showed leucopenia ($2{\cdot}5 \times 10^9$/l), an ESR of 22 mm/h and a CRP value of 25 mg/l. There was no eosinophilia. Investigations for bacteria, fungi, parasites and viruses were negative. Biopsy of a subcutaneous nodule showed panniculitis, with histiocytes exhibiting haemophagocytosis. There was no response to broad-spectrum antibiotics, but an initial response to prednisone 40 mg/day. Reduction of the prednisone

Table 1. Clinical and laboratory features

Patient	1	2	3	4
Age at onset (years)	61	23	17	20
Sex	F	M	M	F
Location of skin nodules	Trunk, left arm	Face, trunk, arms, legs	Face, trunk, arms, legs	Face, trunk, arms, legs
Fever	+	+	+	+
Hepatomegaly	−	+	+	−
Splenomegaly	+	+	+	−
Peripheral adenopathy	−	−	+	−
Serosal effusion	−	Pleural	Ascites	−
Weight loss (kg)	20	25	5	0
Anaemia	+	+	+	+
Leucopenia	+	+	+	+
Thrombocytopenia	+	+	+	−
High serum levels of liver enzymes	+	+	+	−
Cytophagic histiocytes in extracutaneous organs	Bone marrow	Spleen	Bone marrow, spleen	−

dose, however, resulted in new episodes of fever and the appearance of more subcutaneous nodules. A course of diethylcarbamazine was started because it was suspected that she might be suffering from filariasis, but there was no response.

In April 1990 her fever was still present (temperature up to 39·0°C), and there were subcutaneous nodules on her face and trunk, arms and legs. There was no lymphadenopathy or hepatosplenomegaly. She had mild anaemia (Hb 11·0 g/dl) and persistent leucopenia, but the platelet count remained normal. CRP concentration, routine chemistry and chest X-ray were normal. There was mild polyclonal hypergammaglobulinaemia. Tests for rheumatoid factor and antinuclear antibodies were negative. Transaminase, alkaline phosphatase, lysozyme and ACE levels were normal. On computed scans of the abdomen, the small intestine was distended, and its wall was thicker than normal. This was also seen on an X-ray of the small intestine. Gastroscopy and colonoscopy were normal, and scanning with ^{111}In-labelled granulocytes showed no pathological uptake. Laparoscopy was normal, as was a liver biopsy.

In August 1990 azathioprine 50 mg/day was added to her medication, with a good response. However, discontinuation of azathioprine 6 months later resulted in a relapse. On her latest follow-up visit she was in remission, taking azathioprine 50 mg/day and prednisone 5 mg/day. Her skin shows atrophy and scars at the sites of previous nodules.

The main clinical and laboratory features of the four patients are summarized in Table 1.

Discussion

The histiocytic disorders form a spectrum of diseases varying from benign regional histiocytoses to malignant histiocytosis, which is rapidly fatal.[8–10] CHP is considered to be a low-grade form of malignant histiocytosis with involvement initially confined to the skin.[2,11] The major clinical features are recurrent fever, multiple panniculitic lesions, anaemia and leucopenia. Later there may be involvement of the liver and spleen, serosal effusions, mucosal ulceration, haemorrhages and weight loss. Histologically the panniculitis is lobular, with marked fat necrosis, haemorrhage, and an inflammatory infiltrate composed mainly of benign-appearing histiocytes, but also of lymphocytes and occasional neutrophils. Pathognomonic of CHP are 'bean-bag' cells, i.e. large histiocytes which have phagocytosed leucocytes and erythrocytes. Morphologically benign histiocytes may also be found in the bone marrow, lymph nodes, liver, spleen, myocardium and gastrointestinal tract.

Careful exclusion of infections is mandatory in patients with CHP. Reactive haemophagocytosis has been seen in association with viral, bacterial, fungal and parasitic infections.[12–14] The viruses most often implicated in the development of haemophagocytosis are viruses of the herpes group, particularly cytomegalovirus and Epstein–Barr virus.[12] The virus-associated haemophagocytic syndrome has been described primarily in immunocompromized patients, such as recipients of renal transplants.[12] Recently, a similar form of haemophagocytic syndrome has been described in association with human immunodeficiency virus infection.[15] Despite extensive investigations we were unable to demonstrate any active infection in our patients.

CHP has been decribed in a few patients with B- or T-cell lymphomas.[16–18] Alegre and Winkelmann[3] observed a large number of benign-appearing T cells in the cutaneous lesions of patients with CHP, and suggested that CHP may be a reactive process in response to an underlying T-cell proliferation, or a T-cell disease itself. Using T-cell receptor rearrangement techniques Coupe *et al.*[4] showed clonal T-cell proliferation in a patient who had CHP but no clinical or morphological evidence of lymphoma. It is conceivable that cytokines released by proliferating polyclonal or monoclonal T lymphocytes could lead to the activation of histiocytes which results in the clinical manifestations of CHP.

We detected high serum TNF-α concentrations in one patient. Macrophages are among the main producers of cytokines such as TNF-α. TNF has been shown to induce fever, anaemia and cachexia when injected into experimental animals,[19] and it is possible that some of the clinical manifestations of CHP are explained by overproduction of TNF. TNF is also known to stimulate the production of acute-phase reactants, such as CRP, from hepatic cells. However, all our patients had normal or only slightly elevated serum CRP levels. In agreement with the original report of patients with CHP,[2] and with descriptions of patients with malignant histiocytosis, the erythrocyte sedimentation rates in our patients were either normal or only slightly raised.

The five patients originally described by Crotty and Winkelmann[2] died from liver failure and haemorrhage after a disease duration of 6 months–10 years. There have been several subsequent reports of similar patients,[3] but a number of patients with CHP and a benign clinical course have also been described.[4,20–22] Certain clinical and laboratory features are associated with a poor prognosis: bleeding, mucosal ulcers, lymphadenopathy, fever, hepatosplenomegaly, serosal effusions, anaemia, leucopenia, coagulopathy, and hypocalcaemia.[21] Although our patients had many clinical features indicating a poor prognosis (Table 1), they did not develop progressive liver failure or spontaneous bleeding. Three of them responded to treatment and are alive 2·5–4·5 years after diagnosis. The fourth patient showed an initial response, but died of an infection associated with treatment-induced leucopenia.

Various drugs have been tried for the treatment of CHP, but the results have usually been disappointing. We noticed a great variation in our patients' response to treatment. One patient achieved remission with corticosteroids alone, and another did not respond until azathioprine had been added to the corticosteroid medication. Two required a combination of cytostatic drugs (Bleo-CHOP); in one of these patients corticosteroids and a single cytostatic drug had been unsuccessful. Our results support the view presented by Alegre and Winkelmann[3] that early treatment with cytostatic drugs is advisable in patients with the more serious forms of CHP.

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British Journal of Dermatology (1992) **127**, 641–644.

Diffuse primary cutaneous amyloidosis

J.F.BOURKE, J.BERTH-JONES AND D.A.BURNS
Department of Dermatology, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

Accepted for publication 22 June 1992

Summary A case of extensive primary cutaneous amyloidosis exhibiting both macular and lichenoid lesions is reported. Lesions were arranged in a distinctive linear pattern covering much of the trunk and limbs, in places following Blaschko's lines. The coexistence of macular and lichenoid lesions suggests that this is an unusual variant of biphasic cutaneous amyloidosis.

Primary cutaneous amyloidosis is defined as cutaneous amyloidosis in the absence of other systemic or dermatological disease.

It may be classified into three principal categories: macular, lichenoid (lichen amyloidosus) and nodular.[1] The nodular form is rare. It is characterized by the development of single or multiple nodules, most commonly involving the trunk or limbs. It results from a localized plasma cell dyscrasia. Immunohistochemistry of these lesions reveals immunoglobulin light chains.[2]

Macular and lichenoid varieties are clinically and histologically distinct from the nodular form. It has been proposed that the amyloid in these varieties arises from degenerate epidermal keratinocytes.[3] They have characteristic epidemiological profiles and are occasionally inherited.[4,5] Macular amyloidosis is commoner in Central and South America, India and the Middle East, whereas lichen amyloidosus is more common among the Chinese.[1] Macular amyloidosis is seen on the limbs and the upper trunk, where hyperpigmented macules 2–3 mm in diameter may coalesce into larger confluent lesions. Lichen amyloidosus develops more commonly on the shins and consists of discrete hyperkeratotic hyperpigmented papules which may coalesce into plaques.

Case report

A 46-year-old white Caucasian male presented with a 6-year history of a mildly pruritic rash which first appeared on his right shoulder and subsequently became gradually more widespread. He had no relevant past medical history and there was no family history of any similar condition.

Examination revealed a maculopapular, variably pigmented dermatosis involving the trunk and all four limbs. There were areas of telangiectasia, purpura, scaling and hyperkeratosis. Lichenoid lesions were prominent on the legs. The pattern varied from reticulate over the thighs (Fig. 1) to whorled on the back (Fig. 2) with a well-demarcated linear arrangement over areas

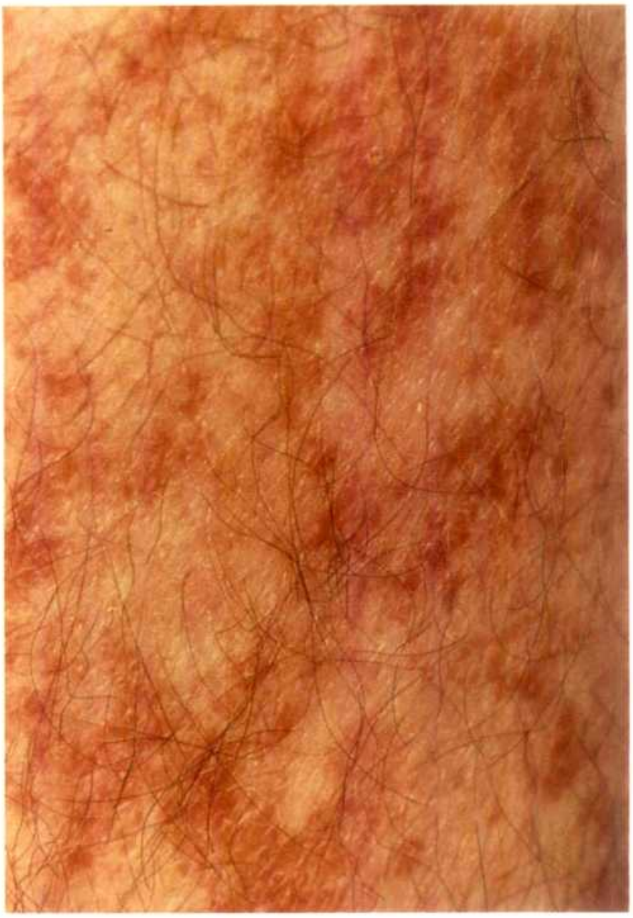

Figure 1. Lichenoid lesions in a reticulate pattern on the right thigh.

Correspondence: Dr J.F.Bourke.

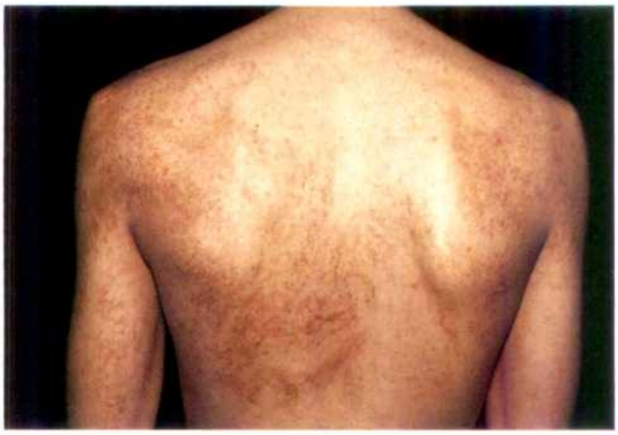

Figure 2. Macular lesions in a whorled and arcuate distribution on the back.

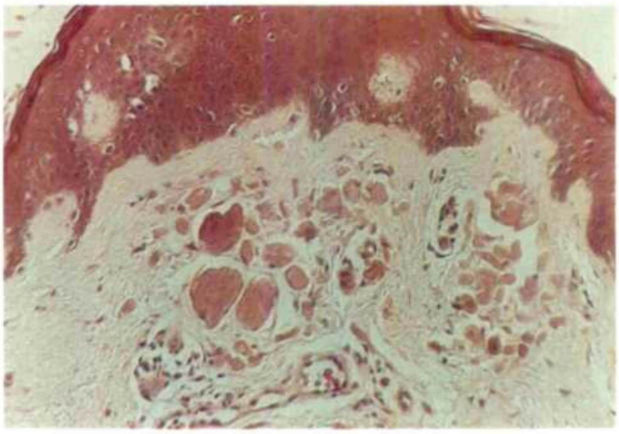

Figure 4. Eosinophilic amyloid globules in the upper dermis (haematoxylin and eosin ×40).

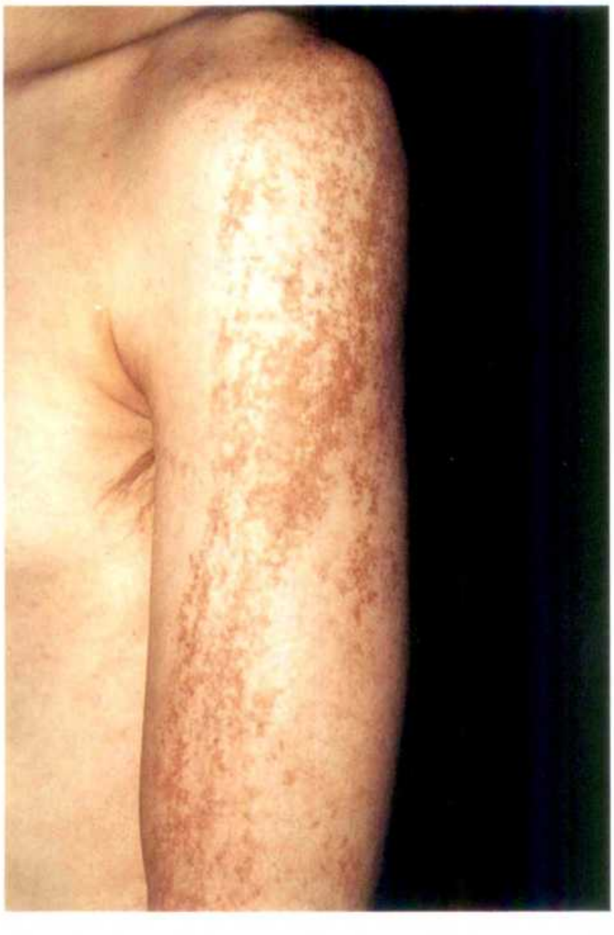

Figure 3. Linear lesions following Blaschko's lines on the arms.

of the back and upper limbs (Figs. 2 and 3) which appeared to follow Blaschko's lines.

Histology revealed round eosinophilic globules in the upper dermis (Fig. 4) which stained positively with Congo red and demonstrated apple-green birefringence when viewed under polarized light. Masses of amyloid fibrils were seen on electron microscopy (Fig. 5). Immunohistochemical staining was negative for kappa and lambda light chains and amyloid A protein, but strongly positive for cytokeratins (Fig. 6). All other investigations (full blood count, biochemical profile, plasma protein electrophoresis, rheumatoid factor, antinuclear factor, creatinine clearance, urine protein excretion and examination for Bence–Jones protein, ECG, chest X-ray, abdominal ultrasound, bone marrow aspirate, rectal biopsy) were normal or negative. There was no evidence of systemic amyloidosis.

Over a 3-year follow-up period, the appearance and distribution of the rash have not changed.

Discussion

Our patient presented with both macular and lichenoid lesions coexisting in an extensive and unusual distribution.

Extensive variants of macular amyloidosis have been described, and arcuate, swirled or reticulate hyperpigmentation has been documented in some cases. Partington *et al.*[6] described a family with hereditary cutaneous amyloidosis of variable severity which was very similar in distribution to our case. Individual family members were found to have linear, swirled, streaked or reticulate lesions, sometimes following Blaschko's lines.

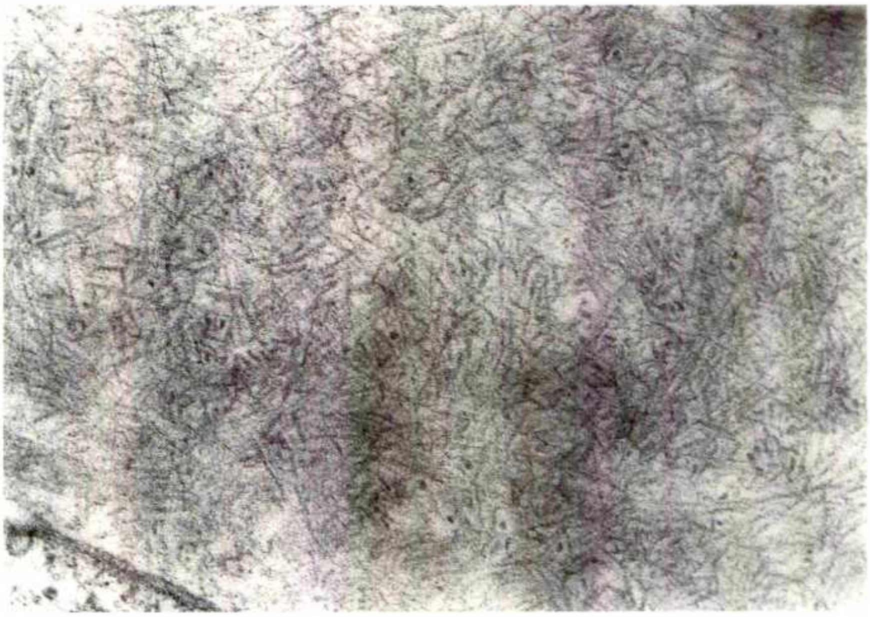

Figure 5. Electron microscopy revealing masses of amyloid fibrils (× 99,000).

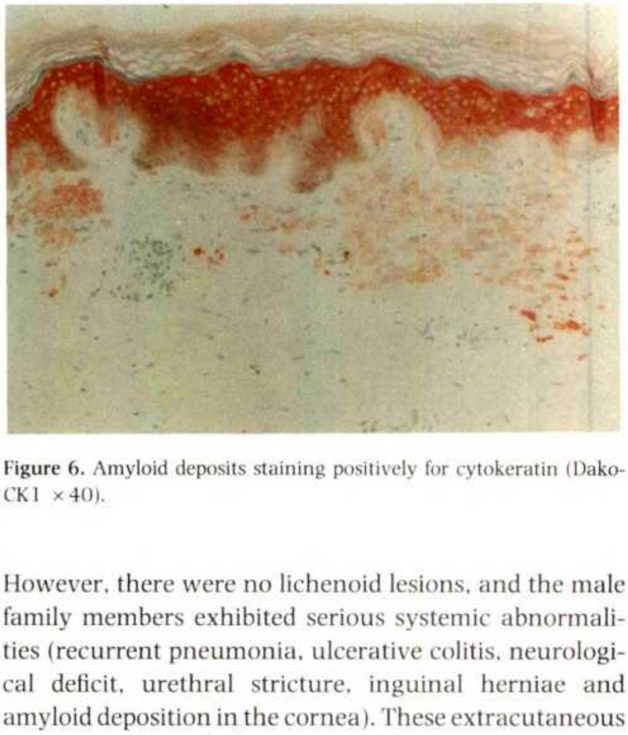

Figure 6. Amyloid deposits staining positively for cytokeratin (Dako-CK1 × 40).

However, there were no lichenoid lesions, and the male family members exhibited serious systemic abnormalities (recurrent pneumonia, ulcerative colitis, neurological deficit, urethral stricture, inguinal herniae and amyloid deposition in the cornea). These extracutaneous features and family history were absent in our patient. Zaynoun *et al.*[7] described a 43-year-old Syrian man with diffuse macular hyperpigmentation over the face, trunk and limbs. The lesions over the trunk and proximal extremities showed a reticulate and rippled pattern, but not a linear arrangement.

Eng *et al.*[8] described a swirled hypopigmented pattern in twins with macular amyloidosis which resembled the truncal pattern of hyperpigmentation in our patient, but histology did not reveal amyloid in these areas and the authors attributed the pigmentary change to an independent process. These cases were further complicated by cardiopulmonary anomalies (pulmonary fibrosis, bronchiectasis, and a fibrotic pulmonary valve in one case, and prolonged Q–T interval in the other).

Black and Maibach[9] described a 17-year-old Caucasian girl with unilateral hyperpigmented macular amyloidosis affecting the medial aspect of the left thigh, lower leg, ankle, and left lower abdomen. The abdominal component of the eruption was arcuate in appearance but in other areas the macules were poorly defined. No linear or reticulate lesions were present.

Extensive cases of lichen amyloidosus have also been reported. Le Boit and Greene[10] described symmetrical reticulate abdominal hyperpigmentation in twins with lichen amyloidosus, both of whom had other systemic abnormalities (mental retardation and mitral valve prolapse). Clarke[11] described a 42-year-old man with extensive lichen amyloidosus involving the forearms, buttocks, thighs and left shin. The lesions, unlike our case, were not arranged in any particular pattern and the syndrome was complicated by granulomatous hepatitis and poor intellect.

Our patient differs from these extensive cases of amyloidosis in that he has developed both macular and lichenoid lesions.

Another unusual variant of primary cutaneous amyloidosis, so-called poikiloderma-like cutaneous amyloidosis was originally described by Marchionini and John,[12] and recently reviewed by Ogino and Tanaka.[13] These patients exhibit poikiloderma-like changes, mainly on the extremities, and lichenoid lesions on the shins. Some of these cases also suffered from other disorders and Ogino and Tanaka proposed the existence of a subclass of patients with light sensitivity, short stature and blister formation or palmoplantar hyperkeratosis (the poikiloderma-like cutaneous amyloidosis syndrome). Although some of the lesions on our patient's body loosely resemble poikiloderma because of the presence of telangiectasia and variable pigmentation, the overall pattern is linear and is distinct from the cases previously described. In addition our patient does not have any of the other systemic features sometimes associated with this form of amyloidosis.

Coexistence of macular and lichenoid lesions in cutaneous amyloidosis is well recognized. Brownstein *et al.*[14] proposed the title 'biphasic primary cutaneous amyloidosis' for these patients, and extensive cases of biphasic amyloidosis have been reported from Thailand, India and Spain.[15–17] Piamphongsant and Kullavanijaya[15] described four patients with widespread amyloidosis sparing only the palms and soles. These patients had predominantly macular lesions on the trunk and upper limbs, with lichenoid lesions and guttate hypomelanosis on the shins. Similar cases, but with less extensive involvement, have also been described.[16,17] Our case differs in distribution, but not morphology, from those of Piamphongsant and Kullavanijaya and therefore probably represents an extensive variant of biphasic primary cutaneous amyloidosis characterized by a pattern which is in some areas reticulate and in others follows Blaschko's lines. To our knowledge, this clinical picture has not been described previously.

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Acquired tufted angioma showing spontaneous regression

T.MIYAMOTO, M.MIHARA,* E.MISHIMA,* Y.HAGARI* AND S.SHIMAO*

Division of Dermatology, Tsuyama Central Hospital, Tsuyama, Japan
**Department of Dermatology, Tottori University School of Medicine, Yonago, Japan*

Accepted for publication 5 June 1992

Summary We report a 62-year-old woman with acquired tufted angioma. Several scattered reddish nodules were present on the neck and upper chest. During a follow-up period of 6 months, some of the lesions showed transient spontaneous regression and one disappeared completely. Electron microscopy revealed that a few tumour cells contained Weibel–Palade bodies in their cytoplasm.

In 1989, Wilson Jones[3] described 20 patients with an unusual form of angiomatous proliferation, which occurred most commonly on the neck and upper trunk, predominantly in the young. Because of the characteristic histological findings of small, circumscribed angiomatous tufts and lobules scattered in the dermis, the lesions were designated 'tufted angioma'. Despite the progressive spread of the angiomas, the lesions appear to be benign, and malignant transformation has not been encountered.[1–3,5,6] Similar cases have been reported as 'angioblastoma' (Nakagawa)[4,7–13] in Japan.

We report the case of an elderly woman with lesions showing histological features typical of acquired tufted angioma, in whom the lesions underwent transient spontaneous regression or complete resolution.

Case report

In February 1990, a 62-year-old Japanese woman presented to our hospital, complaining of lesions on her chest and neck. The lesions had been present for 12 months. They had enlarged slowly, and were slightly tender.

Examination revealed several oval, bright-red, slightly tender nodules scattered on the right side of the neck, both sides of the upper chest, and the left clavicular region (Fig. 1). These nodules were attached to the skin, but not to the underlying subcutaneous tissue and muscle (Fig. 2a).

Laboratory findings, including full blood count, total serum protein, and urinalysis, were within normal limits.

Excision biopsy of a nodule on the right side of the neck was performed under local anaesthesia with 1% lignocaine. Four weeks later, the remaining nodules became slightly less prominent and non-tender. After 4 months, they had flattened, and there was brown pigmentation of the overlying skin (Fig. 2b). However, after a further 2 months, three nodules enlarged, and were subsequently excised. By this time, the nodule on the left clavicular region had disappeared completely.

Histology

The first excision biopsy specimen showed a normal epidermis. The dermis contained many scattered tumour masses which were arranged in a lobular fashion (Fig. 3). The tumour cells formed numerous slit-like capillary lumina, some of which contained red blood cells. The cells had clear cytoplasm and an oval nucleus with mild hyperchromasia (Fig. 4a). Silver staining revealed a meshwork of reticular fibres surrounding vessels and

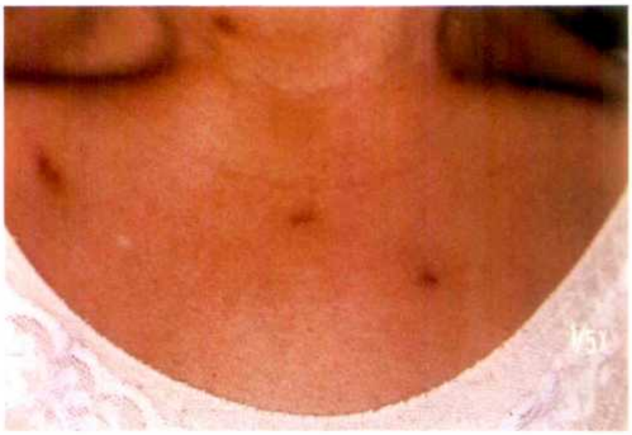
Figure 1. There are five nodules on the neck and upper chest.

Correspondence: Dr T.Miyamoto, Division of Dermatology, Tsuyama Central Hospital, Nikaimachi 67, Tsuyama 708, Japan.

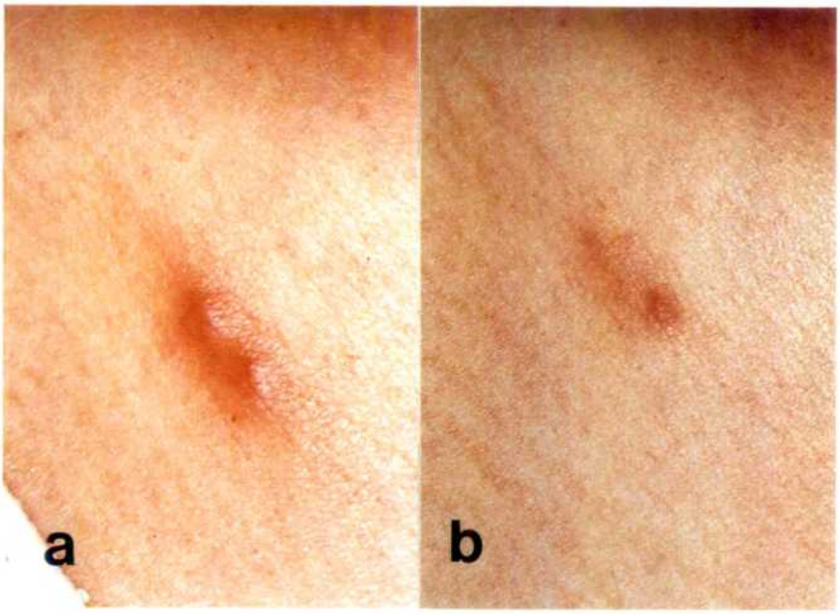

Figure 2. The nodule on the right clavicular region. (a) at the time of presentation; (b) 4 months later.

tumour cell nests. From the mid- to the lower dermis the tumour lobules were surrounded by thick connective tissue composed of collagen fibres. Most of the collagen fibres were mature and partially hyalinized, but some were immature. In the specimens excised 6 months later, the surrounding collagen fibres penetrated the tumour lobules. Comparison with the first specimen showed that the capillary lumina were more dilated (Fig. 4b).

Electron microscopy of the later biopsy specimens revealed that the cytoplasm of the tumour cells contained several types of organelle, including mitochondria, lysosomes, and vacuoles, as well as a number of microfilaments. Some tumour cells formed a capillary lumen (Fig. 5). A few tumour cells contained Weibel–Palade bodies (Fig. 5), but crystalline lamellae[13,14] were not seen.

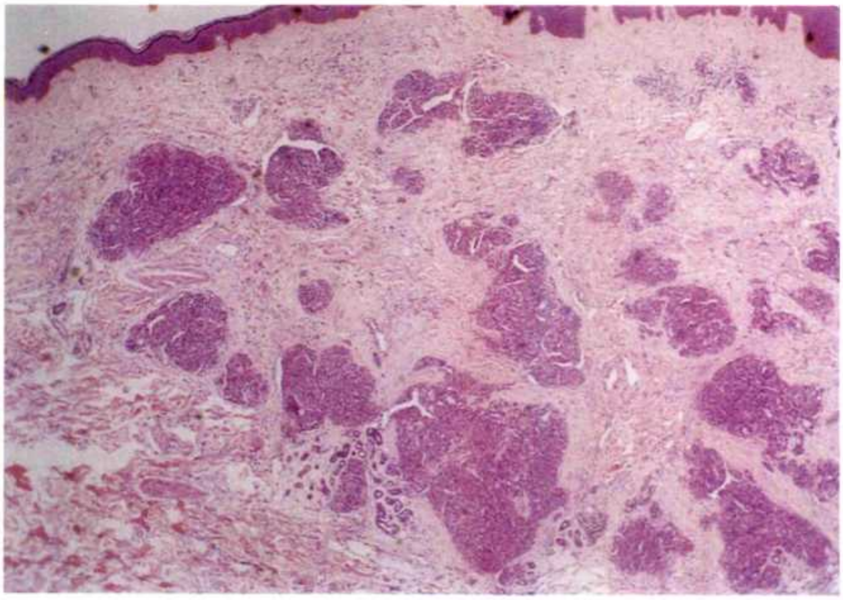

Figure 3. The dermis contains many scattered tumour masses arranged in a lobular fashion (haematoxylin and eosin × 50).

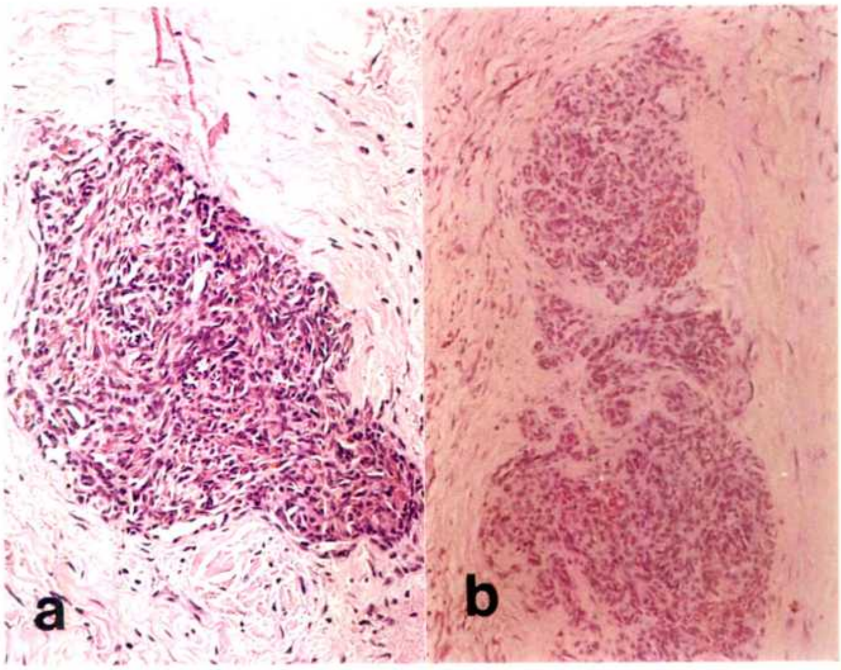

Figure 4. (a) The first biopsy specimen (haematoxylin and eosin × 370); (b) the second specimen (haematoxylin and eosin × 200). Mature and immature collagen fibres surround the tumour lobules and, in the second specimen, some have penetrated the tumour lobules.

Immunohistochemically, positivity for factor VIII-related antigen (FVIII-RA) and *Ulex europaeus* agglutinin I (UEAI) was limited to the cells lining the larger and better formed vascular channels. The majority of the cells in the proliferating, i.e. poorly canalized, capillaries, were negative.

Discussion

The clinical and histological features of our case were similar to those of acquired tufted angioma described by Wilson Jones.[3] However, our case presented three unusual features.

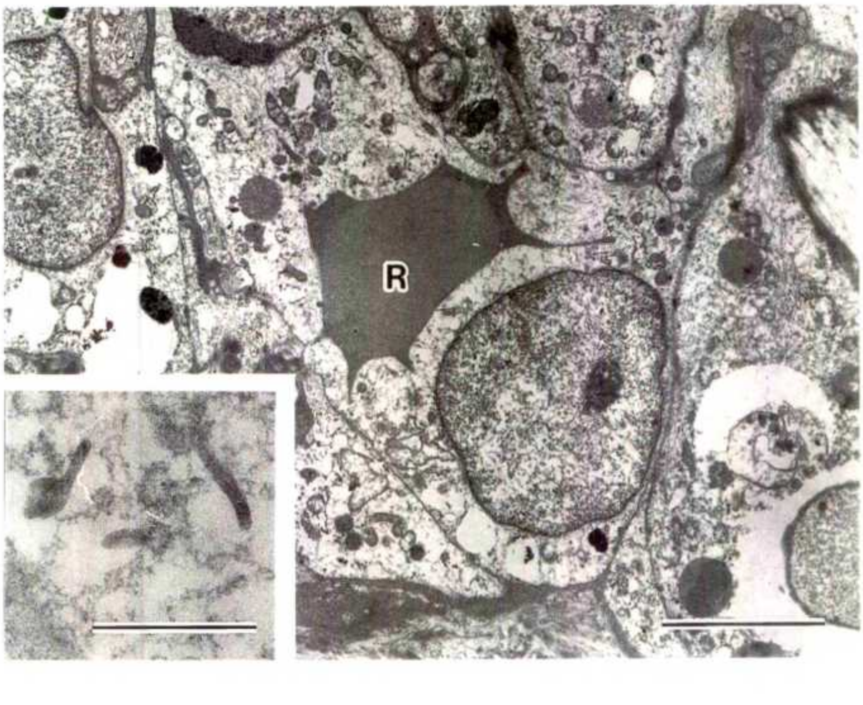

Figure 5. Electron micrograph. R, red blood cell (× 4200; scale bar 5 μm). Inset: a few tumour cells contained Weibel–Palade bodies (× 42,000; scale bar 0·5 μm).

1. The lesions occurred in an elderly woman, and the individual lesions were widely dispersed on the neck, upper chest and left clavicular region. The lesions of tufted angioma usually develop in the young, predominantly within the first 5 years of life, and they tend to be localized.[3,7,13]

2. During a 6-month follow-up period, three out of four nodules showed transient spontaneous regression, and one disappeared completely. Histology of the lesions excised 6 months after presentation revealed that the surrounding collagen fibres had penetrated the tumour lobules. This finding suggests that the tumour tissues were being replaced by the connective tissue, and is probably histological evidence of the spontaneous regression of the lesions. In the case of infants with angioblastoma (Nakagawa), spontaneous complete involution occasionally occurs.[7,9,10] Thus, there is a possibility that acquired tufted angioma may resolve spontaneously, even in patients who are elderly at the onset of the condition.

3. Electron microscopy revealed Weibel–Palade bodies in the tumour cells, but most of these cells were not immunohistochemically positive for UEA-1 or FVIII-RA. This discrepancy between the electron microscopic and the immunohistochemical findings may have been due to the immaturity of the endothelial cells.

Acknowledgement

The technical assistance of Mr K.Yamada, Mrs T. Kobayashi and Mrs I.Yoshii is greatly appreciated.

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Do pigmented naevi in albinism provide evidence of tyrosinase positivity?

M.AKIYAMA,*† H.SHIMIZU,* M.SUGIURA† AND T.NISHIKAWA*

**Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160, Japan*

†Department of Dermatology, Shimizu City Hospital, 1231 Miyakami, Shimizu, Shizuoka 424, Japan

Accepted for publication 10 April 1992

Summary We investigated a 4-year-old Japanese boy with oculocutaneous albinism who had a solitary pigmented mole measuring 5 mm in diameter on his back. An electron microscopic tyrosine incubation test and a DOPA reaction test clearly demonstrated the presence of tyrosinase activity in the patient's hypopigmented skin. The presence of tyrosinase activity was confirmed by tests on hair bulb samples. Histopathological evidence showed that the mole was a typical compound cellular naevus with melanin pigmentation. Although no reports to date have focused on the relationship between pigmented naevi in albinism and tyrosinase activity, our findings suggest that the occurrence of pigmented naevi in an albino may indicate the presence of tyrosinase activity.

Oculocutaneous albinism is an inherited disorder of the melanin pigmentary system characterized by a congenital decrease in, or absence of melanin synthesis in the skin, hair, and eyes. The most common types of oculocutaneous albinism are the tyrosinase-positive and -negative varieties, classified according to tyrosinase activity.[1] Some cases of tyrosinase-positive albinism have a good prognosis, with pigmentation frequently appearing as the patients grow older. In tyrosinase-negative albinism, depigmentation is complete and no pigmentation occurs throughout life.[2] Patients with this severe type of albinism, especially non-Caucasian patients, can experience many serious problems, and their skin colour may lead to social discrimination.[3]

Knowing the tyrosinase classification of a child with albinism is also helpful in prenatal diagnosis or counselling. Prenatal diagnosis is not indicated in tyrosinase-positive albinism, because the prognosis is good. It is helpful to know if a first affected child has tyrosinase-negative albinism, because the tyrosine incubation test and DOPA reaction test can then be used as aids in prenatal diagnosis.[4–6] We investigated a 4-year-old Japanese boy with albinism, who had a solitary pigmented mole, using a variety of methods including the DOPA reaction test at an ultrastructural level. We describe our detailed investigation of this case, including the results of the electron microscopic DOPA reaction test, and discuss the possibility that a pigmented naevus in albinism is an indicator of tyrosinase positivity.

Case report

A 4-year-old Japanese boy with oculocutaneous albinism, and his mother, were referred to us. His mother was pregnant, and was hoping for prenatal diagnostic tests. The boy's parents and grandparents showed normal pigmentation, and he had no known history of other diseases. We decided to perform prenatal diagnosis in case the proband suffered from tyrosinase-negative albinism.

On physical examination, we noted that the child's skin was completely white, and his hair was pale blonde (Fig. 1). His eyes were pale red–brown, and the pupils

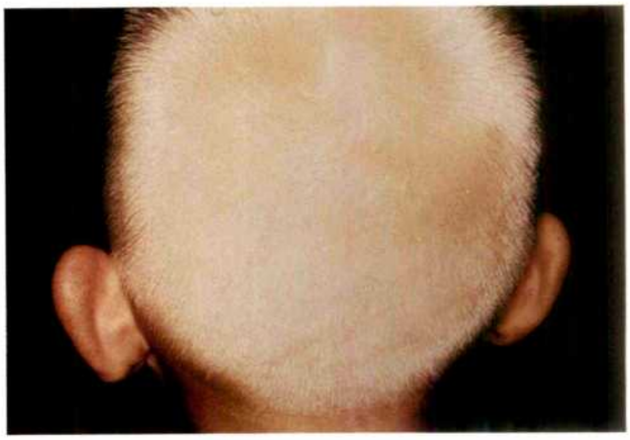

Figure 1. Scalp of the patient. The skin is white and all his hair is pale gold in colour.

Correspondence: Professor T.Nishikawa, Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160, Japan

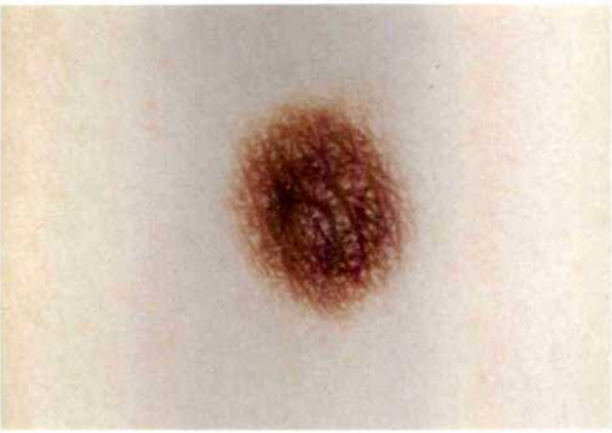

Figure 2. A mole, 5 mm in diameter, on the right of the patient's back.

appeared to be red in colour. Ophthalmological examination revealed horizontal nystagmus and a total absence of pigment in the fundi. Visual acuity was normal. We found a round, brownish, slightly elevated mole, measuring 5 mm in diameter, on the right side of his back (Fig. 2). We excised the mole, with an area of surrounding hypopigmented skin, and processed it for light microscopic study. Hypopigmented skin was also processed for electron microscopy. Fontana–Masson staining was performed for detection of melanin pigment by light microscopy. The mole was composed of cuboidal or oval cells with round nuclei in clusters in the upper dermis and at the dermo-epidermal junction. Some cells, particularly in the superficial area, contained melanin. Melanophages were also observed in the upper dermis (Fig. 3). These findings indicated a compound cellular naevus. Light microscopy of haematoxylin and eosin-stained sections revealed no melanin pigment in the basal layer of the epidermis of the hypopigmented skin. Fontana–Masson's stain did not show any melanin pigment. These findings were compatible with a diagnosis of albinism.

Electron microscopy revealed a small number of melanosomes in the cytoplasm of some of the basal cells. Almost all the melanosomes were in stage II or III (Fig. 4a).

We performed the DOPA reaction test and the tyrosine incubation test[7] on the specimens from hypopigmented skin, and on hair bulb specimens, to determine the patient's type of albinism. We also evaluated the hypopigmented skin with an electron microscopic DOPA reaction test, as previously described.[8–10] A moderate

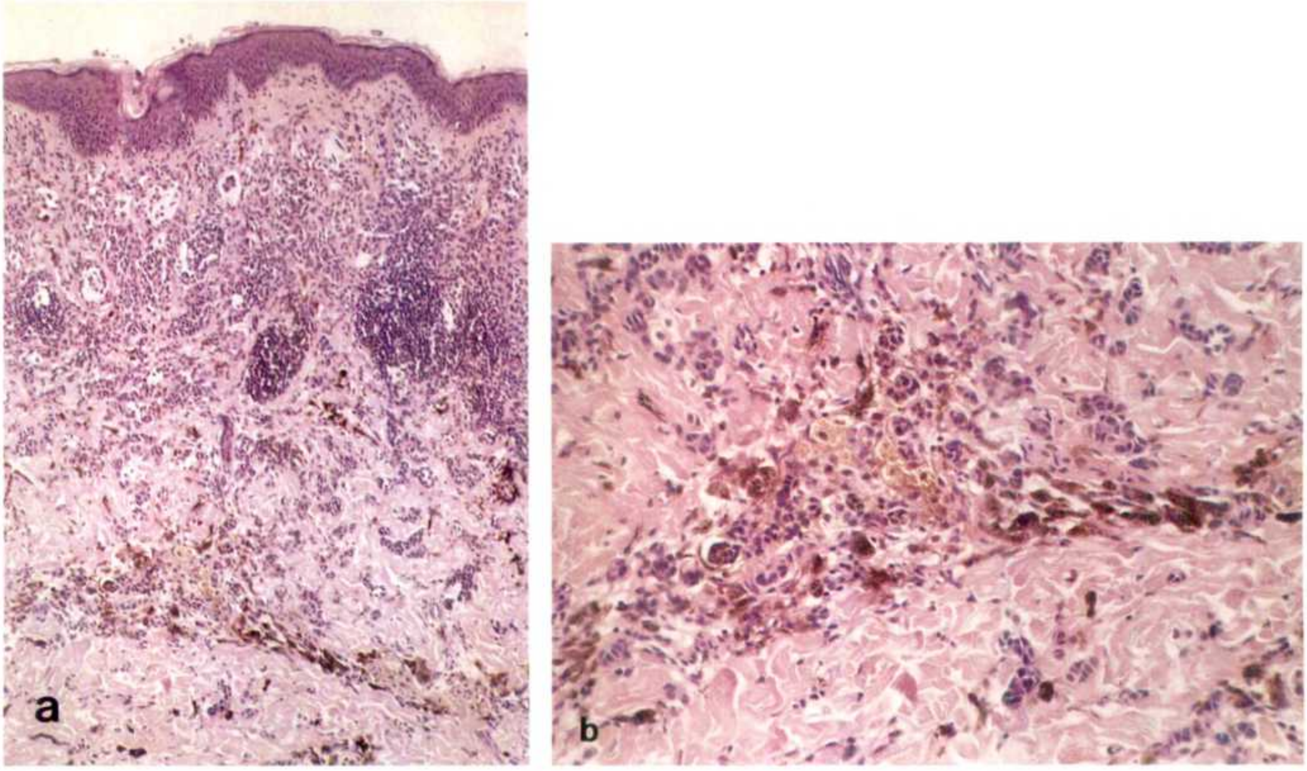

Figure 3. (a) Low-power photomicrograph of the mole, showing clusters of naevus cells (haematoxylin and eosin, × 50). (b) Some of the naevus cells contain melanin pigment (haematoxylin and eosin, × 100).

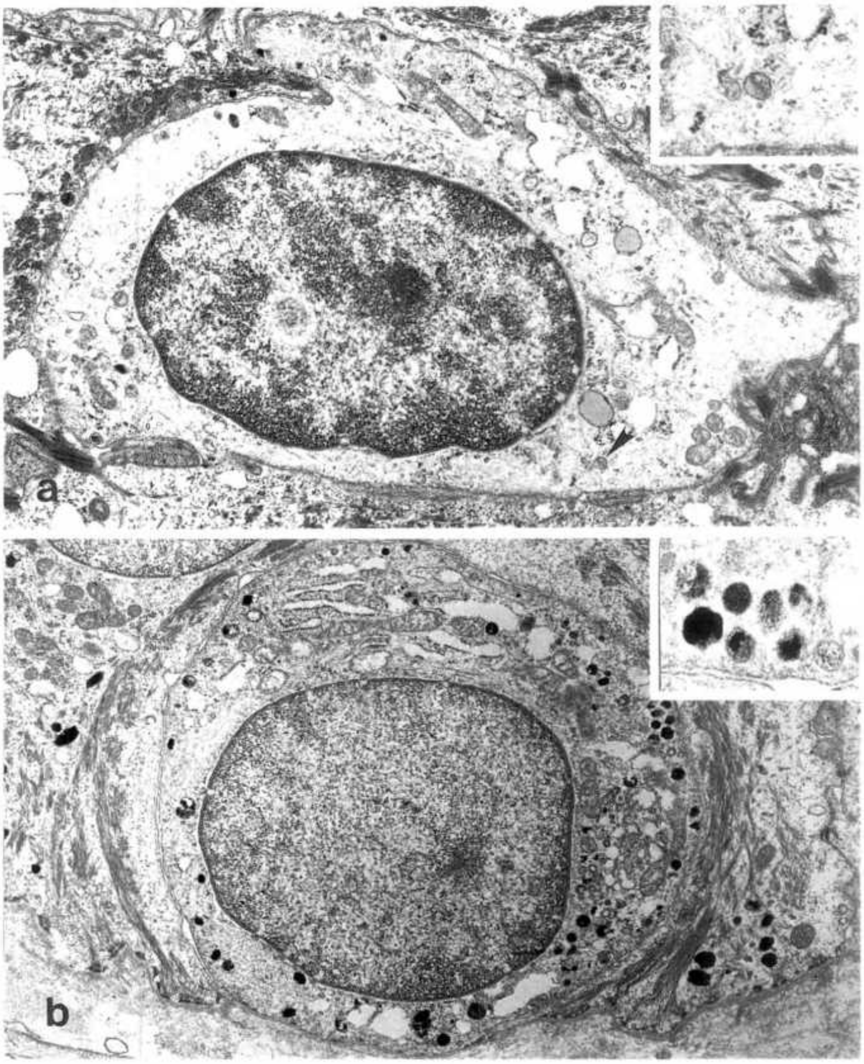

Figure 4. (a) A melanocyte in the hypopigmented skin of the patient. Without DOPA reaction, pigmented melanosomes are difficult to find in the cytoplasm. Only a few granules recognizable as premature melanosomes are seen (arrowhead) (enlarged in inset) (× 16 000; inset, × 34 000). (b) With DOPA reaction, a large number of fully pigmented melanosomes are seen in the cytoplasm of a melanocyte, as well as in keratinocytes. Inset: melanosomes in stage II–IV in the melanocyte (× 15 000; inset, × 48 000).

amount of pigment was seen in the basal layer of the depigmented skin on light microscopy after both the DOPA reaction and tyrosine incubation tests. At the ultrastructural level, a striking increase in the number of melanosomes, many of which were in the fully pigmented stage IV, was observed in the cytoplasm of the cells of the basal and prickle cell layers. We observed many fully pigmented melanosomes in the cytoplasm of a melanocyte (Fig. 4b).

Before incubation in the tyrosine or DOPA solution, the patient's hair bulb was almost transparent. After incubation in the tyrosine solution, the hair bulb became pigmented, appearing dark brown (Fig. 5a). A similar change in colour was observed after treatment with the DOPA solution. Samples incubated in phosphate-buffered saline without tyrosine or DOPA exhibited no pigmentation (Fig. 5b). These findings clearly demonstrated the presence of tyrosinase activity in this case.

Discussion

Although there are reports of pigmented naevi occurring in albinism,[11–16] only one of these describes a solitary pigmented nevus.[16] However, that case also had a lesion of lentigo maligna; the authors emphasized that melanoma development depends on the presence of melanocytes, but not necessarily on melanogenesis.[16] The relationship between a solitary pigmented naevus and tyrosinase activity was not discussed. To our knowledge, our case report is the first to suggest that a solitary pigmented naevus is a sign of the presence of tyrosinase activity in albinism. When we reviewed reported cases of

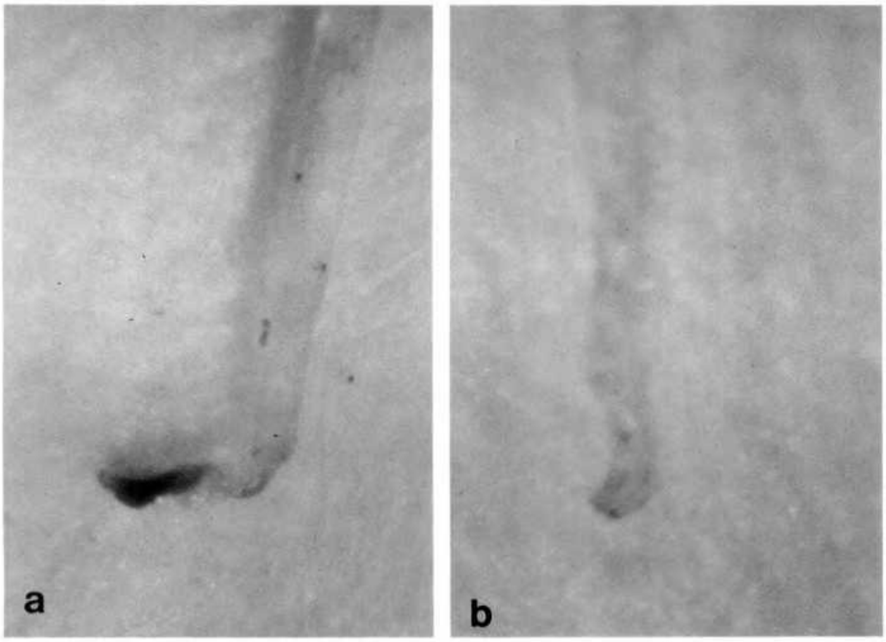

Figure 5. (a) After incubation in tyrosine solution, the hair bulb of the patient became pigmented. (b) Control specimen showed no change in colour.

pigmented naevi in albinism, we found that they have been described only in cases of tyrosinase-positive albinism.[14-16]

Recent reports have indicated that the albino phenotype of some patients with tyrosinase-negative albinism is a consequence of inactivated tyrosinase caused by a mutation in the tyrosinase gene.[17-20] If the albino phenotype of all cases of tyrosinase-negative albinism is caused by a mutation of the tyrosinase gene, no pigmentation can be observed on any part of the body, even in a mole, unless chimerism or back-mutation of the tyrosinase gene occurred in some parts of the body. To date, there are no reports of such chimerism or back-mutation. In keeping with this point of view, pigmented naevi represent important signs of tyrosinase positivity in albinism. Tyrosinase from hair bulbs of tyrosinase-positive albino patients has been reported to be identical to the normal enzyme.[21,22] The basic defect that causes hypopigmentation in tyrosinase-positive albinism is unknown, and a possible abnormality in the distal eumelanin pathway of melanin synthesis has been suggested.[22] At present, the explanation for the existence of pigmented naevi in tyrosinase-positive albinism is unclear, although two hypotheses may be proposed: the existence of a different synthesis or transport pathway in the substrate of tyrosinase, or the reversion of the naevus cells to normal phenotype in melanin synthesis.

Several methods can identify the type of albinism, such as the tyrosine incubation test and DOPA reaction test using a hair bulb or skin specimen.[7-10] However, the presence of a solitary pigmented naevus can provide additional evidence of tyrosinase positivity, and we therefore suggest that examination of a patient with albinism should include a search for such naevi.

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Correspondence

Time course of activity of topical 8-methoxypsoralen on palmoplantar skin

Sɪʀ, Topical PUVA was first used to treat palmoplantar psoriasis in 1974[1] and since then a number of studies have been published[2–8] indicating variable results. In these studies treatment schedules have varied considerably with regard to the method of psoralen application and the time interval between psoralen application and UVA irradiation. Beneficial results were reported in six of the studies[1–6] and in these the psoralen was applied 1–2 h before UVA irradiation. In the two studies[7,8] in which no benefit was reported the psoralen was applied just prior to irradiation in all or most of the cases.

It has been shown previously that following a trioxsalen bath the maximum sensitization of normal skin to UVA radiation occurred immediately afterwards, and that this was followed by a significant decrease within 1 h. In comparison, trioxsalen painting produced maximum sensitization 1–2 h after application, with an effectiveness of about 50% of that produced with bathing.[9] We report the results of a study to determine the optimum time for UVA irradiation on palmoplantar skin folowing the application to topical psoralen by immersion.

Table 1. UVA-irradiated sites exhibiting erythema at various times after psoralen soaking (min)

Subject No.	Minutes												
	0	10	20	30	40	50	60	70	80	90	100	110	120
Left hand													
1	0	0	1	1	1	1	1	1	1	1	1	1	1
2	0	0	0	1	1	1	1	1	1	0	0	0	0
3	1	1	1	1	1	1	1	1	1	1	1	1	1
4	0	1	0	1	0	1	0	1	1	0	1	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	1	1	1	0	1	1	1	0	0
Right hand													
1	0	0	1	1	1	1	0	1	1	1	1	1	1
2	0	0	0	1	1	1	1	1	1	1	0	1	0
3	0	1	1	1	1	1	1	1	1	1	1	1	1
4	0	1	0	0	0	0	1	1	1	1	1	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	1	0	0	0	0	0	1	1	0
Total number of erythematous sites on both hands													
	1	4	4	9	8	8	7	8	9	7	8	6	4
Left foot													
1	0	0	1	0	1	0	1	1	0	1	1	1	1
2	0	0	1	1	1	1	1	1	1	0	1	1	1
3	0	1	1	1	1	1	1	1	1	1	1	1	1
4	0	0	0	0	1	0	1	0	1	0	0	0	0
5	0	0	0	0	1	0	0	0	0	0	0	0	0
6	0	0	0	0	0	1	1	0	0	0	0	0	0
Right foot													
1	0	0	0	0	1	0	1	1	1	1	1	1	1
2	0	0	1	1	1	1	1	1	1	0	1	1	1
3	0	1	1	1	1	1	1	1	1	1	1	1	1
4	0	0	0	1	1	0	1	1	0	1	0	1	0
5	0	0	0	0	1	0	0	0	0	0	0	0	0
6	0	0	0	0	0	1	0	0	0	0	0	1	0
Total number of erythematous sites on both feet													
	0	2	5	5	10	6	9	7	6	5	6	8	6
Total number erythematous sites on both hands and both feet													
	1	6	9	14	18	14	16	15	15	12	14	14	10

Six healthy volunteers (four female, two male; age range 24–58) with no history of palmoplantar skin disease were recruited following approval from the local Ethics Committee. An irradiation monochromator set to a central wavelength of 350 nm with a 30 nm full bandwidth at half maximum intensity was used. A liquid-filled light guide conducted the radiation from the exit slit of the monochromator to the patient's skin. The irradiance at the end of the light guide was 160 mW/cm^2. Spectroradiometric measurements of the erythemally effective irradiance as a fraction of the total ultraviolet irradiance confirmed that this use of the irradiation monochromator resulted in a close approximation to the spectral power distribution of UVA fluorescent lamps commonly used in PUVA units (e.g. Philips TL09).

One millilitre of Deltapsoralen® bath lotion 1·12% (Delta Pharmaceuticals Ltd, Ireland) was diluted in 2 l of warm water. The hands and feet were immersed in the diluted psoralen solution for 15 min and then dried. A template made from thin adhesive cardboard with 13 holes (6 mm diameter) in a grid pattern was applied to the centre of the palms and soles to facilitate accurate irradiation of the test sites, and again at the time of reading to help identify the previously irradiated sites. Irradiation of each hand and foot with a dose of 5 J/cm^2 began immediately after soaking, and was repeated at 10-min intervals for a total of 2 h. With an irradiance of 160 mW/cm^2 the exposure time at each site was 31 s. Sites chosen for irradiation at each time interval were randomized between subjects, but kept constant between the right and the left sides. The sites were examined at 72 h simply for the presence or absence of erythema, rather than attempting to assess the degree of erythema using an ordinal scale (i.e. 1+, 2+, etc.). A chi-squared test was used to examine differences in the time course of erythemal sensitivity between the left and right hand, left and right foot, and both hands and feet.

The presence or absence of erythema at each of the irradiation sites is indicated in Table 1. In every subject the number of sites on which erythema was seen was not significantly different ($P>0{\cdot}2$) between left and right hand or left and right foot. There was no significant difference between the time course in either hand or foot, or between hands and feet ($P>0{\cdot}9$ in every case). Consequently the responses observed at each of the irradiation times were summated to give the number of sites (out of a possible total of 24) in which erythema was observed at 72 h. These data form the bottom row of Table 1. It can be seen that the maximum number of sites in which erythema was seen occurred when the extremities were irradiated 40 min after removal from the psoralen solution. A chi-squared test indicated no significant difference ($P>0{\cdot}05$) between erythemal sensitivity at this time and that at times between 30 and 110 min. However, the skin was significantly more sensitive at 40 min than when irradiated at 20 ($P<0{\cdot}05$), 10 ($P<0{\cdot}01$) and 0 min ($P<0{\cdot}001$) after removal.

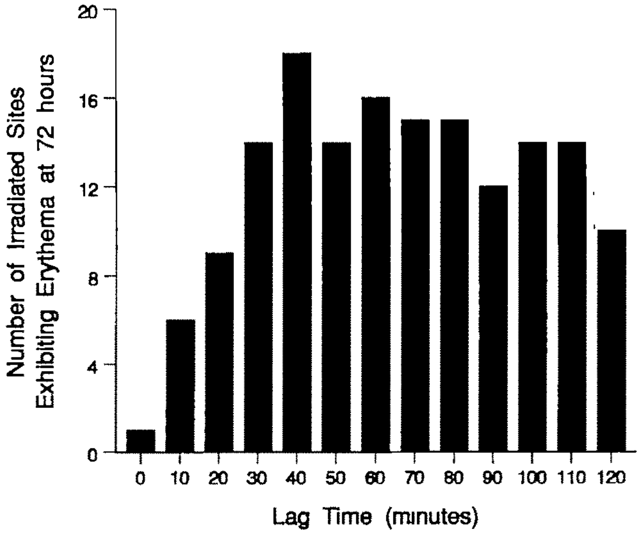

Figure 1. The number of sites (out of a possible 24) on which erythema was present at 72 h after irradiation, for different waiting periods (lag time) between finishing soaking and beginning irradiation.

The results indicate that there is a lag time of approximately 40 min after soaking the hands and feet in psoralen solution before maximum UVA sensitivity is reached, and that this is followed by a sustained sensitivity for a further hour or so (Fig. 1). This observation may be explained by the kinetics of the drug in palmoplantar skin compared with elsewhere on the body. The stratum corneum forms the principal barrier to the absorption of psoralen through the epidermis[10] and on the palms and soles it is 400–600 μm thick compared with about 10 μm elsewhere on the body. This increased thickness of the stratum corneum also provides a significant drug reservoir which may account for the sustained activity.

It has previously been demonstrated that the vehicle significantly affects the penetration of the psoralen through the epidermis, and that the greatest penetration occurs with solutions and emulsions.[10] In this study the hands and feet were immersed in the dilute psoralen solution in an attempt to avoid variations that may occur with applications such as painting. Soaking also ensures that the epidermis is fully hydrated because the degree of hydration is known to affect the permeability of the stratum corneum.

We conclude that the maximum erythemal sensitivity of palmoplantar skin to UVA radiation following soaking in psoralen solution occurs between 30 and 110 min. It would seem reasonable to suppose that the therapeutic response of palmoplantar psoriasis follows a similar time course, although this remains to be demonstrated.

Dermatology Department, J.Konya
Royal Infirmary, Sunderland SR2 7JE B.L.Diffey*
**Regional Medical Physics Department,* T.C.Hindson
Dryburn Hospital, Durham DH1 5TW

Correspondence: Dr B.L.Diffey.

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Localized trichorrhexis nodosa

SIR, Trichorrhexis nodosa (TN) is the most common hair shaft anomaly, with distinctive clinical and structural features. Scanning-electron microscopy is an excellent tool to investigate structural anomalies of the hair shaft.[1] The primary cause of TN is trauma, although several hereditary syndromes exist which predispose to weakness of the hair shaft. Apart from the congenital form, three clinical varieties of acquired TN may be distinguished: proximal, distal and circumscribed types (Table 1).[2] The following case report documents clinically, and by light microscopy and scanning-electron microscopy, a case of localized TN without an underlying dermatosis or a history of physical or chemical hair trauma.

A 37-year-old, otherwise healthy man noted a localized hair change on the left and right retroauricular regions at the age of 16. There was no history of any form of trauma to the hair. The patient realized that the hairs in these circumscribed areas broke very easily. The first medical record concerning these hair changes was in 1976. Examination in January 1992 revealed two 4 × 3 cm symmetrical areas of abnormal hair in the retroauricular regions. On closer inspection, the hair shafts were broken, and small whitish nodules were visible (Fig. 1). Several hairs had multiple nodules which were more prominent on the distal half of the hair shaft, although some nodules could also be seen on the proximal part. The hair colour was the same as that on the rest of the scalp, and the underlying skin was normal. The patient had slight androgenetic alopecia but otherwise the hair was normal. Light microscopy showed the typical features of TN and scanning-electron microscopy demonstrated the characteristic nodular swelling with fractured cortical fibres and absent cuticle at the site of the fracture (Fig. 2).

Table 1. Acquired trichorrhexis nodosa[2,7]

Proximal TN	Blacks; history of hair straightening
Distal TN	Whites and Orientals; history of physical and chemical trauma induced by hair care
Localized TN	With underlying skin diseases; trichotillomania; spontaneous; woolly hair naevus

The localized type of TN occurs on the scalp, moustache, beard or pubic area, depending on the site of repeated trauma.[3,4] Chemical injury, such as prolonged treatment with selenium, may be involved in acquired TN.[5] Touraine and Clerfeuille[6] stated that the condition may affect one area, or several areas simultaneously. They emphasized that the hair adjacent to the patches of localized TN is absolutely normal. They also mentioned that the underlying skin is usually normal, although seborrhoeic dermatitis or chronic erythrokeratoderma may be present. Acquired localized TN may develop in infancy or adult life. The course is chronic, and no specific treatment exists, apart from avoidance of trauma.

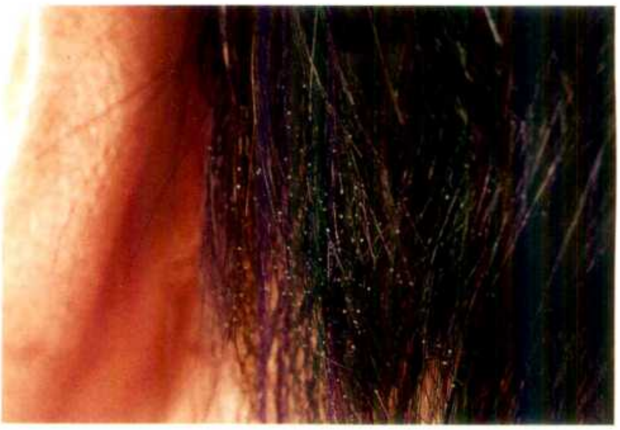

Figure 1. Close view of trichorrhexis nodosa. Several hair shafts end with a whitish nodule.

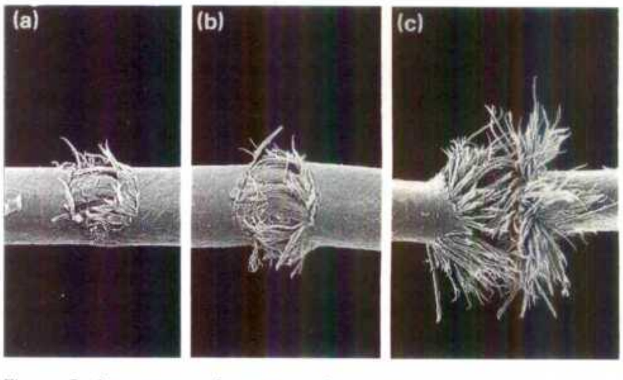

Figure 2. A sequence of scanning-electron micrographs showing (a) development of nodular swelling, (b) absence of the cuticle, (c) fracture of the cortical fibres (× 200).

Differential diagnoses include dandruff, hair casts and pediculosis. Diagnosis is based on the typical clinical presentation, and changes visible on light microscopy and scanning-electron microscopy. Excessive physical and cosmetic trauma should be avoided to minimize weathering of the hair shafts.

Department of Dermatology,
University Hospital,
4031 Basel,
Switzerland;
**Laboratory for Scanning*
Electron Microscopy,
University of Basel,
Bernoullistrasse 32,
4056 Basel, Switzerland

P.H.ITIN
G.MATTARELLI
A.J.BIRCHER
E.ZUBERBÜHLER*
R.GUGGENHEIM*

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Clear eyewear for PUVA patients

SIR, Induction of cataract is a potential adverse effect associated with PUVA therapy. Studies in experimental animals have shown that exposure of the intra-ocular lens to UV within 12–24 h of administration of psoralen causes the formation of photoproducts which accumulate within the lens.[1 2] However, provided the animals are kept in the dark during this period, 8-MOP diffuses freely out of the lens. In guinea-pigs, the action spectrum for the effect was found primarily in the UVA region, with maximum efficiency between 320 and 340 nm, and no effect at wavelengths longer than 380 nm.[2] 8-MOP has also been observed in a human lens for at least 12 h following ingestion of 0·75 mg/kg of 8-MOP.[3] Consequently, patients receiving PUVA are advised to use protective eyewear for 24 h after taking psoralen.

A survey of 58 pairs of sunglasses used by PUVA patients showed that many were satisfactory, but 33% failed to meet proposed transmission limits.[4] Moreover, the disadvantages associated with wearing tinted sunglasses were recognized and therefore a further study was undertaken into the UV-blocking properties of clear lenses, and a number of lenses or lens coatings were found to be suitable for use by PUVA patients.[5] These were included in a list of recommended eyewear issued by the British Association of Dermatologists.[6] This guidance was particularly apt because product standards for sunglasses issued by British, American and European standards institutions are not adequate for PUVA protective eyewear.

However, the list was not intended to be exclusive and we have recently examined more samples of clear lenses with UV-blocking properties and determined their transmission as described previously.[5] These lenses are described in Table 1 and their transmission spectra are shown in Figure 1. The lenses all had little or no tint. Their transmission in the UV region is shown in Table 2, in comparison with the limits which we proposed earlier.[4,5] The Vision Express and Spectron coatings which we tested are both acceptable and may be applied to plastic prescription lenses. The Unispec and Auer products are

Table 2. Transmission in the UV region for UV-blocking, clear lenses compared with the recommended limit

	Transmission (%)				
Wavelength (nm)	Vision Express	Spectron	Unispec	Auer	Recommended Limit
390	0·1	0·3	2·0	14·8	10
380	0·1	<0·1	<0·1	0·5	5
370	<0·1	<0·1	<0·1	0 4	2
<360	<0·1	<0·1	<0·1	0·3	1

Table 1. Lenses examined in the present study

Lens	Description	Supplier
Vision Express	Inland Dipdye coating from Vision Express, St. Enoch Centre, Glasgow	Vision Express U.K. Ltd, 4 Longwall Avenue, Queens Drive Estate, Nottingham NG2 1NA, U.K.
Spectron	Spectron 400 coating produced by Melson Wingate	Melson Wingate Ltd, 31 Abbott Road, Bournemouth BH9 1EZ, U.K.
Unispec	Unispec 6000 clear lens general purpose safety eyewear	Pulsafe Ltd, Lichfield Road Ind. Est., Tamworth, Staffs. B79 7XL, U.K.
Auer	Auer Perspecta-Fit clear lens general purpose safety eyewear	Auer Perspecta, Lyskaer 13, DK 2730, Harlow, Denmark

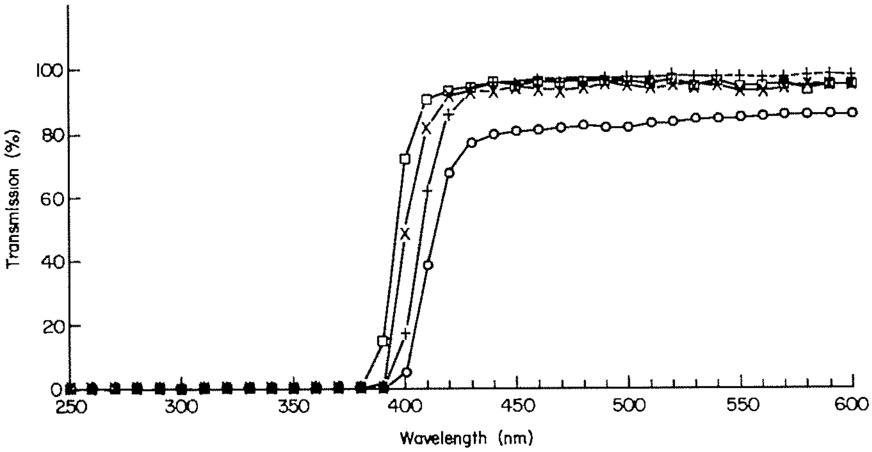

Figure 1. Transmission spectra of clear lenses with UV-blocking characteristics ○, Vision Express; +, Spectron; ×, Unispec; □, Auer.

Table 3. Clear lenses or lens coatings recommended for use by PUVA patients

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Lenses treated with Orcolite UV 400
Essilor Orma UVX lenses
Sola UV Gard 400 lenses
Rodenstock Perfalit Lambda 400 lenses
Lenses coated with Vision Express Inland Dipdye
Lenses coated with Melson Wingate Spectra 400
Pulsafe clear Unispec 6000 eyewear

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general-purpose, safety eyewear incorporating clear lenses. They are inexpensive (approximately £5 per pair) and are acceptable cosmetically, particularly if side-shields are removed. Although the Auer eyewear confers significant UV protection, it does not comply with the recommended transmission limit of 10% at 390 nm.

Clear lenses or lens coatings which meet our criteria of acceptability are listed in Table 3, which shows products which we reported on previously,[5] in addition to those described in the present study. Polaroid polarizing sunglasses have acceptable UV-transmission properties[5] but do not have clear lenses and so are not included in Table 3.

*West of Scotland Health Boards,
Department of Clinical Physics and
Bio-Engineering,
11 West Graham Street,
Glasgow G4 9LF, U.K.
†Department of Dermatology,
Western Infirmary,
Glasgow G11 6NT, U.K.

H.Moseley*
W.Perkins†

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Erythema dyschromicum perstans in an HIV antibody-positive man

Sir, Ashy dermatosis, otherwise known as erythema dyschromicum perstans,[1] was first decribed by Ramirez in 1957.[2] It is a chronic skin disorder, characterized by hyperpigmented macules of various sizes on the trunk, face and extremities. The palms, soles, scalp, nails and mucous membranes are spared.[3] The lesions are usually asymptomatic, although mild pruritus may occur.[4]

Ashy dermatosis appears most prevalent in Latin Americans,[3,5] but there have been occasional reports in patients of Caucasian origin.[6–9] The aetiology of this condition remains obscure, although there have been reports of an association with ammonium nitrate,[10] and whipworm infestation.[11] It has been speculated that an unidentified environmental pollutant may be the cause in certain predisposed individuals.[12] Although there has been a report of ashy dermatosis being present following a seroconversion illness with the human immunodeficiency virus (HIV) in a child,[13] we present the first case occurring in a known HIV antibody-positive adult.

A 41-year-old homosexual of Chinese origin, who was known to have been HIV antibody-positive for 54 months, presented with a 6-week history of increasing truncal pigmentation. He had no illness defining the acquired immunodeficiency syndrome, and was receiving dideoxyinosine 375 mg b.d. as antiretroviral therapy. As his CD4 count was only $173/\text{mm}^3$, he had started Fansidar® (pyrimethamine 25 mg, sulfadoxine 500 mg) as prophylaxis against the development of *Pneumocystis carinii* pneumonia 8 weeks previously. He was receiving no other medication.

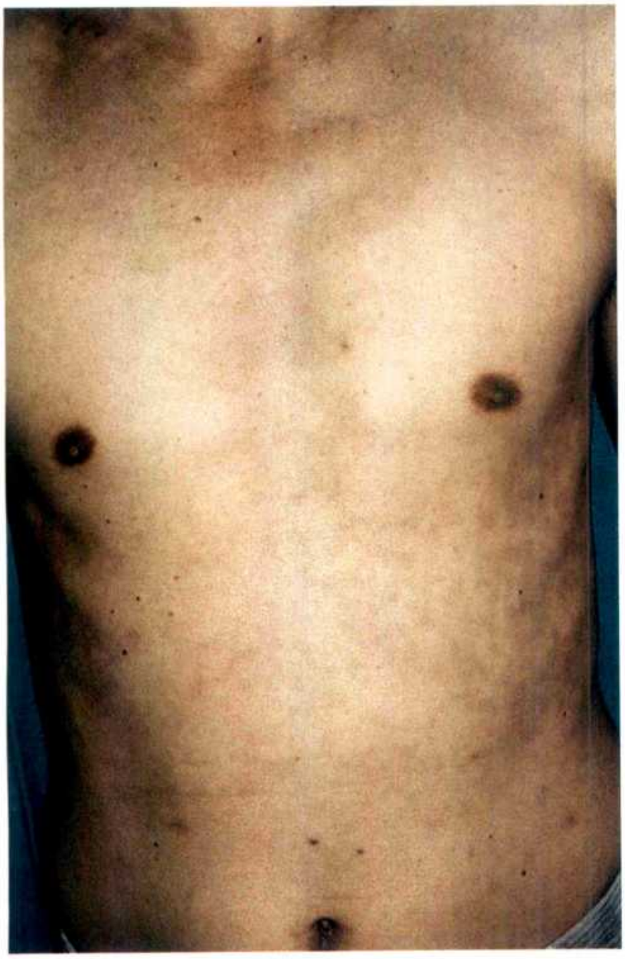

Figure 1. Macular hyperpigmented areas on the trunk.

On examination there were small macular areas of gunmetal grey hyperpigmentation (Fig. 1). These were maximal centrally, on his mid-back, flanks and chest. There was no increase in pigmentation on his limbs or mucosa. Histological examination of a skin biopsy showed mildly acanthotic skin with occasional vacuolated cells, particularly in the lower epidermis. A mild perivascular chronic inflammatory infiltrate was present in the superficial dermis, and there were profuse dermal melanophages. A diagnosis of erythema dyschromicum perstans was made.

Cutaneous abnormalities are common in HIV-infected individuals,[14] and if followed over several months, almost 100% of such patients manifest skin disease.[15] However, ashy dermatosis has previously been reported in only one individual with HIV.[13] This is surprising, as down-regulation of the immune system may be an important component in the aetiology of this condition.[16]

There is no recognized treatment for this condition, but many have been tried, including antibiotics, steroids, isoniazid, griseofulvin, chloroquine, sunscreens, keratolytics and psychotherapy,[5,7,11,17,18] and a recent study reported marked improvement in seven out of eight patients treated with clofazimine.[16]

AIDS Unit,
Westminster Hospital,
London SW1P 2AP, U.K.

M.R.NELSON
A.G.LAWRENCE
R.C.D.STAUGHTON
B.G.GAZZARD

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News and Notices

2nd Dermatopathologic Colloquium of Giessen, 13–14 March 1993, Giessen, Germany

The second Dermatopathologic Colloquium of Giessen will take place on 13–14 March 1993 at Giessen, Germany. The Chairman, and principal speaker, will be Professor A.B.Ackerman (New York University). For further information please contact: Dr M.Nilles and Dr W.Weyers, Congress Secretaries, Department of Dermatology and Andrology, Hautklinik, Gaffkystr. 14, 6300 Giessen, Germany. Telephone 0641-7023520, Fax 0641-7023542.

1993 ESDR Scientific Meeting, 3–6 April 1993, Amsterdam

The 1993 ESDR Scientific Meeting will take place on 3–6 April 1993 in Amsterdam, The Netherlands. This meeting will be held in the RAI Congress Centre in Amsterdam. For further information please contact: Professor Dr R.Willemze, Department of Dermatology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Telephone (31)-20-548-3496, Fax (31)-20-548-2848.

Melanoma '93, 6–7 May 1993, Brighton

The Royal College of Pathologists and the Melanoma Study Group, with the support of the SE Thames Regional Health Authority and the Brighton Health Authority, present a conference to examine melanoma and other pigmented skin lesions, which will include formal presentations and slide seminars. Histology specimens will be available for microscopy. The Faculty will include: R.L.Barnhill, Boston; K.Blessing, Aberdeen; M.G.Cooke, Guildford; D.E.Elder, Philadelphia; N.Kirkham, Brighton; J.A.Newton, London; N.P.Smith, London and W.J.Mooi, Amsterdam. Biopsy cases may be submitted in advance of the meeting for circulation to a panel of experts, prior to their discussion. The programme will be suitable for both trainees and consultants in histopathology, dermatology and dermatopathology, and will be of interest to other specialists involved in the diagnosis or management of patients with pigmented skin lesions. Abstracts for free presentations related to the diagnosis or treatment of melanoma will be accepted. A 1-day introductory course for those inexperienced in this area of skin pathology will precede the main meeting. Numbers for this course will be limited. A boxed collection of sample biopsies will be circulated beforehand to registrants. Hotel accommodation and secure car parking will be available at discount rates. The conference takes place during the Brighton festival. For further information please contact: Mrs P.Newland, Melanoma '93, Department of Histopathology, Royal Sussex County Hospital, Brighton BN2 5BE, U.K. Fax 0273-600182.

Joint Annual Meeting of The British Association of Dermatologists and The Canadian Dermatology Association, Oxford, 6–10 July 1993

The 73rd Annual Meeting of the British Association of Dermatologists will be a joint meeting with the Canadian Dermatology Association under the joint presidency of Dr William Gerstein (Canada) and Dr Terence Ryan (UK).

The meeting will be held in the Academic Centre at the John Radcliffe Hospital, Oxford from Tuesday, 6th July to Saturday, 10th July 1993.

Abstracts of papers and posters are invited for consideration by a Joint Abstract Selection Committee and those accepted as papers will be allotted 10 minutes for presentation plus time for discussion. Abstracts must be submitted on the official forms, which are available from Miss Michelle Hardwicke, BAD Conference Office, 3 St. Andrew's Place, London NW1 4LB.

The closing date for receipt of abstracts is Friday, 15 January 1993 but applicants should note that the Scientific Committee will be administered by the Canadian Dermatology Association and extra time, therefore, should be allowed for postage to Canada.

Instead of the usual clinical/CPC session held on Saturday morning, there will be a 'Celebration of Dermatology' to be held in the Churchill Hospital, Oxford. Further details will be published in the autumn edition of the BAD Newsletter.

Dermatovenereology Course 1993. STD for Trainee Dermatologists, 22 January 1993, The Royal London Hospital, Whitechapel, London

A 1-day course on Dermatovenereology will be held at the Royal London Hospital, Whitechapel, London. This will be conducted by specialists in both fields and will include six lectures covering clinical diagnosis and management of most aspects of sexually transmitted disorders, including dermatoses affecting the genitalia, tumours, bacterial and non-bacterial infections, with special emphasis on HIV infection. There will also be a demonstration of up-to-date diagnostic techniques used in STD clinics. The course fee is £50.00, and includes coffee/tea and lunch. The number of places is limited. For further information please contact: Dr Rino Cerio, Consultant Dermatologist, The Royal London Hospital, Whitechapel, London E1 1BB. Telephone 071-377-7000, ext. 2491.

A 5-day residential training course in contact dermatitis, University of Birmingham, 26–30 April 1993

This course will cover practical and theoretical aspects of contact dermatitis. In addition to patch test clinics, there is a structured lecture programme, with additional visits to industrial sites which present potential dermatological hazards. For further information please contact: Joyce Blake, Course Co-ordinator, The Institute of Occupational Health, University of Birmingham, Edgbaston, Birmingham B15 2TT. Telephone 021-414-6021.

Diploma of Dermatopathology

The second sitting of the examination for the Diploma of Dermatopathology will take place in the Spring of 1993. This Diploma is organized jointly by the Royal College of Pathologists and the British Association of Dermatologists. The closing date for entry for the Spring 1993 examination is 15 January 1993. The written examination will be held on Tuesday 30 March, and those who pass this will be entitled to proceed to the practicals and orals which will be held during the period 10–28 May. All those wishing to sit this examination should obtain an entry form and further details from: Miss Elspeth Evans, Examinations Officer, Royal College of Pathologists, 2 Carlton House Terrace, London SW1 5AF.

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CLINICAL AND EXPERIMENTAL ALLERGY

MONTHLY IN 1992

Journal of the British Society for Allergy and Clinical Immunology

Editors **A.B. Kay** and **S.T. Holgate**

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milligram(s) (10^{-3}g)	mg
microgram(s) (10^{-6}g)	μg
nanogram(s) (10^{-9}g)	ng
picogram(s) (10^{-12}g)	pg
hours(s)	h
minute(s)	min
centimetre(s)	cm
second(s)	s
cubic millimetre(s)	mm^3
millimetre(s)	mm
millicurie(s)	mCi
milliequivalent	mmol
molar	mol/l
millilitre(s)	ml
gravitational acceleration	*g*
micrometre(s)	μm
per cent	%
isotopic mass number placed as	^{131}I

These and other abbreviations and symbols should follow the recommendations of: *Units, Symbols and Abbreviations. A Guide for Biological and Medical Editors and Authors* (1988) The Royal Society of Medicine, London.

References. Only papers closely related to the author's work should be referred to; exhaustive lists should be avoided. References should be given in the Vancouver style, i.e. references appear as superscript numbers in the text, e.g. '. . . our previous report[1] and that of Smith *et al.*[2] . . .', and are brought together numerically in the reference list at the end of the article in order of their first mention in the text. References to articles and papers should mention: (1) name(s) followed by the initials of the author(s), up to four authors: if more than four authors, include first three authors followed by *et al.*; (2) title of paper; (3) title of journal abbreviated in the standard manner; (4) year of publication; (5) volume; (6) page numbers of the article. *Thus*: Cunliffe WJ, Shuster S. The rate of sebum excretion in man. *Br J Dermatol* 1969; **81**: 697–9. References to books and monographs should include: (1) author(s) or editors(s); (2) paper (if necessary) and book titles; (3) edition, volume, etc.; (4) place; (5) publisher; (6) year; (7) page(s) referred to. *Thus*: Beare JM, Wilson Jones E. Necrobiotic disorders. In: *Textbook of Dermatology* (Rook AJ, Wilkinson DS, Ebling FJ, eds), 2nd edn., Vol. 2. Oxford: Blackwell Scientific Publications, 1972; 1066.

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IN PSORIASIS

Changing patients lives

Prescribing Informati
Indication: Mild to modera
plaque psoriasis affecting
to 40% of skin ar
Presentation: 30g and 10
tubes of Dovonex ointment,
micrograms calcipotriol per
in a smooth, whit
preservative-free bas
Dosage and Administratio
Apply twice daily to
affected area. A course
treatment should not l
longer than 6 weeks and
weekly dose should not exce
100g. No experience
children or pregnancy. **Con**
indications: Patients w
known calcium metaboli
disorders. Hypersensitivity
any constituents. **Precautio**
Should not be used on the
Wash hands after applying
ointment. **Drug Interactio**
No experience of concomit
therapy with other topi
antipsoriatic products. **Sid**
effects: Transient lo
irritation. Facial or perio
dermatitis. **Basic N.H.S. Pr**
£7.95 / 30g, £22.85 / 10
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